

Calcium Transport Across
the Chick Small Intestine
as
Facilitated by Vitamin D

by

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Thesis submitted in fulfilment of
the requirements for the degree of
Doctor of Philosophy

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1973

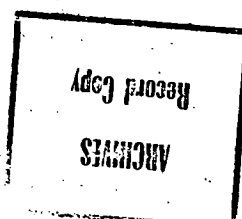


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SUMMARY

The aim of this study was to find an explanation of the enhancing effect of vitamin D on calcium translocation by the small intestine.

Vitamin D had little effect on the distribution or the amount of calcium present in the mucosal cell of chick duodenum no matter what the concentration of the calcium was in the lumen or the time interval the calcium remained in the lumen for periods less than 1 hour. However the turnover of calcium in the cells of vitamin D₃-replete chicks was increased.

The mitochondria were found to contain most of the calcium of the cell when homogenation and fractionation were done at 0°C (Hamilton and Holdsworth, 1969). Inhibitors were sought that would prevent calcium uptake by mitochondria. Most of the inhibitors that did prevent uptake also caused release of endogenous calcium from mitochondria. Some inhibitors that did not cause this release of calcium were only partially effective as inhibitors of calcium uptake by mitochondria. One inhibitor which had the required properties was ruthenium red which not only prevented calcium uptake by mitochondria but would do this in the cell homogenate. However it had a disadvantage in that it precipitated proteins subjected to low centrifugal force. The effect of temperature and inhibitors on homogenation and fractionation was shown to effect the distribution of calcium in the cell, increasing the soluble (cytoplasmic) fraction content to about 1 to 2mM-Ca²⁺ at 22°C or above from 1 to 10μM at 0°C. This increase was also shown when the homogenate was fractionated using the zonal

centrifuge at 28°C.

Vitamin D had been shown to induce the synthesis of a protein that bound calcium (Wasserman and Taylor, 1966). This calcium binding protein was located within the cytoplasmic fraction of the cell when the mucosal cells were homogenized in isotonic sucrose.

This calcium binding protein was found to release calcium from mitochondria at 30°C (Hamilton and Holdsworth, 1970). Other substances, such as adenosine nucleotides and prostaglandin that may be involved in the release of calcium from mitochondria were studied but were found to be without an effect. Only parathyroid hormone was shown to effect the calcium release from mitochondria at 30°C and this release only occurred after 30 minutes incubation.

The calcium binding protein and ruthenium red were found not to effect the uptake or transport of calcium by the mucosal cells. However a bile salt did increase calcium transport of rachitic intestine but did not affect the accumulation or distribution of calcium within the mucosal cells. Ethacrynic acid and temperature were shown to effect a pump at the serosal border of the cell, while vitamin D was shown to have little or no effect on the flux of calcium from plasma to lumen.

A model was proposed for the effect of vitamin D on the translocation of calcium by the chick duodenum mucosal cell. It was postulated that calcium protein binding facilitated diffusion at the microvillus surface and also facilitated the efflux of calcium from the mitochondria to the cytoplasm from where it was actively pumped to the extracellular fluid.

FOREWORDScope and General Plan of Thesis

The work reported in this thesis is mainly concerned with the influence of vitamin D on the subcellular distribution of calcium in the duodenum mucosal cell of the domestic fowl. The work is introduced by a résumé on a number of topics related to and that play a role in calcium metabolism in the body. Specific emphasis has been given to the recent discoveries of the metabolism of vitamin D and identification of calcium binding protein. The remainder of the thesis is divided into seven chapters which deal with experimental methods, experimental results and the final conclusions of these results as proposed in a model for calcium transport across the mucosal cell.

Obligatory Statement

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university and to the best of my knowledge and belief, the thesis contains no copy or paraphrase of material previously published or written by another person, except where due reference is made in the text of this thesis.

Acknowledgements

The work described in this thesis was carried out in the Department of Biochemistry, Medical School, University of Tasmania.

I would like to especially thank Professor E.S. Holdsworth to whom I am gratefully indebted for allowing me to study in his department, for his patience, stimulating and encouraging supervision and for a gift of pure calcium binding protein.

To the following persons, I offer my thanks for help received:-

Mrs. L. Longmore and Mr. J.H. Flowerree; for intelligent technical assistance.

Mr. J.E. Jordan; for friendly and immeasurably helpful technical advice and maintenance of equipment.

Mrs. E. Keenan; for untiring and adept technical assistance.

Dr. E. Neville; for stimulating discussion and advice.

Mrs. L. Nichols and Miss D. Wise; for assistance in preparation of diagrams.

Dr. J.D. Sallis; for stimulating encouragement, helpful advice and for a gift of highly purified Parathyroid Hormone.

Upjohn and Company; for a gift of Prostaglandin E_1 .

Professor R.H. Wasserman; for a gift of pure calcium binding protein.

ABBREVIATIONS USED

ADP	=	adenosine diphosphate
AMP	=	adenosine monophosphate
ATP	=	adenosine triphosphate
ATPase	=	adenosine triphosphatase
APase	=	alkaline phosphatase
BSA	=	bovine serum albumin
CaATPase	=	calcium adenosine triphosphatase
CAMP	=	cyclic (3',5') adenosine monophosphate
CaBP	=	calcium binding protein
CC	=	cholecalciferol
C.P.M.	=	counts per minute
CT	=	calcitonin
DB CAMP	=	dibutyl cyclic (3', 5') adenosine monophosphate
1,25 DHCC	=	1,25 dihydroxycholecalciferol
21,25 DHCC	=	21,25 dihydroxycholecalciferol
24,25 DHCC	=	24,25 dihydroxycholecalciferol
26,25 DHCC	=	26,25 dihydroxycholecalciferol
DHT ₂	=	dihydro tachysterol series 2
DHT ₃	=	dihydro tachysterol series 3
DNA	=	deoxyribonucleic acid
2,4 DNP	=	2,4 dinitrophenol
EC-PD	=	electrochemical potential difference
EDTA	=	ethylene diamine tetra-acetic acid
EGTA	=	ethylene glycol-bis (-amino-ethyl ether) N, N' tetra-acetic acid
25 HCC	=	25 hydroxycholecalciferol
25 HDHT	=	25 hydroxycho tachysterol
25 HDHT ₃	=	25 hydroxysterol series 3
25 HEC	=	25 hydroxyergocalciferol
I.U.	=	international unit
LAPase	=	leucine amino peptidase
NAD	=	nicotinamide-adenine dinucleotide
NADH	=	nicotinamide-adenine dinucleotide reduced

NADP	=	nicotinamide-adenine dinucleotide phosphate
N.E.M.	=	N-ethyl maleimide
o.p. DDD	=	1-(2 chlorophenol)-1-(4 chlorophenol)-2,2 dichloroethane
Pi	=	inorganic phosphate
PPase	=	pyrophosphatase
PGE	=	prostaglandin E ₁
PTE	=	parathyroid extract
PTH	=	parathyroid hormone
PTX	=	parathyroidectomised
RNA	=	ribonucleic acid
TCA	=	tricarboxylic acid cycle
TPTX	=	thyroparathyroidectomised
U.V.	=	ultraviolet

INTRODUCTION

Calcium is the fifth most abundant element comprising 3.6% of the earth's crust. It is an important and major inorganic element in the body. It is present in the diet in the form of insoluble salts, e.g. calcium phosphate, a complex which is formed with proteins in milk, also as soluble salts from vegetables and meat. Calcium is an essential element in a number of processes in the body, e.g. clotting of blood, the activation of enzymes, skeletal processes, contractility of muscle and reactivity of the nervous system. The importance of maintaining homeostatic control of plasma Ca^{2+} concentration is evident from the multifold mechanisms which exist for its regulation, and from the adverse physiological consequences which result from any appreciable departure from the established norm.

The percent Ca^{2+} content of the human body is about 1.6 and the $\text{Ca}^{2+}:\text{PO}_4^{3-}$ ratio is 2.0:1.0. On a fat free basis, the percent Ca^{2+} in the body of an adult ranges from 1.8 - 2.5. (Irving, 1957). The skeleton of a human is approximately 15% of the total body weight, and contains about 99% of the total body Ca^{2+} .

The $\text{Ca}^{2+}:\text{PO}_4^{3-}$ ratio of human bones is similar to the $\text{Ca}^{2+}:\text{PO}_4^{3-}$ ratio of rat bones. In the normal male rat, the ash content of fat-free bone is 63%, and of this 38% is Ca^{2+} and 18.5% P1. The ash content of rat bone has been found to increase from 49% at 21 days to 65% at 100 days after birth. Large variations were found in the individual bones in regard to ash content, but the Ca^{2+} content of the ash appeared to be fairly constant (Irving, 1957).

The Ca^{2+} contents of muscle and brain tissue range from 6.5-24.0 mg/100 g and 8-16 mg/100 g of wet tissue respectively. In various animals the skin content is within the range 31-86 mg/100 g of wet tissue (Irving, 1957). The human liver and spleen Ca^{2+} contents are 6 mg/100 g and 9 mg/100 g of wet tissue respectively.

The normal level of blood Ca^{2+} in humans and many other animal species ranges from 9-11 mg/100 ml of serum. The intracellular fluid is believed to contain very low Ca^{2+} concentration. There are probably four forms of Ca^{2+} in the body fluids (c.f. review by Rasmussen and DeLuca, 1963) (i) protein bound, (ii) complexed, (iii) ionised, and (iv) an electrostatic complex with sulphate and phosphate anions. Normally 50% of the Ca^{2+} in the serum is bound to protein and the other 50% is ultrafilterable mainly as ionic Ca^{2+} . Only a small amount of this ultrafilterable Ca^{2+} is in the complex compounds, and these are thought to be due to citrates, sulphates, and phosphates.

When the serum Ca^{2+} decreases, there is a mobilization of phosphorus from the tissues, which raises the serum phosphorus so that the $[\text{Ca}^{2+}] [\text{HPO}_4^{2-}]$ product remains fairly constant. This product is influenced to a varying degree by a number of factors including vitamin D, PTH and calcitonin. In a normal individual the process of homeostasis controls the level of serum Ca^{2+} , so that its mean value shows deviations of less than 2% (Walser, 1962).

ABSORPTION OF CALCIUM BY THE INTESTINE

The absorption of Ca^{2+} is governed by a number of factors such as age, interaction of Ca^{2+} with other nutrients, adaptation to

alteration in Ca^{2+} uptake, the region of intestine, the presence or absence of bile salts, the Ca^{2+} concentration, parathyroid status, the level of blood Ca^{2+} and of major importance is the vitamin D status. Some of these factors will be reviewed.

(1) The site of calcium absorption in the intestine

The main area of Ca^{2+} absorption occurs in the small intestine, but some absorption can occur in the colon (Lengemann, 1963; Harrison and Harrison, 1969). The ability of the small intestine to transport Ca^{2+} against a concentration gradient is most marked in the duodenum and upper jejunum in animals such as rats (Harrison and Harrison, 1951; Schachter, Dowdle and Schenker, 1960a; Lengemann and Comar, 1961; Cramer, 1965; Krawitt and Schedl, 1968; Halbritter, 1968; Urban and Schedl, 1969a), guinea pigs (Schachter and Rosen, 1959), rabbits and horses (Schryver, Craig, Hintz, Hogan and Lowe, 1970), but the terminal ileum is said to be most active in the golden hamster (Schachter and Rosen, 1959; Schachter, Dowdle, and Schenker, 1960a) and chicken (Sallis and Holdsworth, 1962a; Wasserman, 1962). Webling and Holdsworth (1966) studied Ca^{2+} absorption in chick using loops in vivo and found absorption to be the same in the proximal and middle third of the small intestine, but absorption was less in the distal third (ileum). Also Harnmeyer and DeLuca (1969) reported that in the chick, the duodenum was the main site of absorption, in contrast to that reported by Sallis and Holdsworth (1962a) and Wasserman (1962).

In most animals therefore, it is likely that the upper part of the small intestine is the main site of Ca^{2+} absorption although

studies in vivo in the dog (Cramer, 1965) suggest the ileum may also be important. In man, it was found that the duodenum and jejunum were the most effective in absorption (Birge, Peck, Berman and Whedon, 1969; Wensel, Rick, Brown and Volwiler, 1969).

The most rapid rate of absorption of Ca^{2+} per unit length in the small intestine of the rat and dog has been shown to be just beyond the pylorus, but as the transit time through the duodenum was so short, the largest fraction of Ca^{2+} absorbed was from the more distal intestine (Lengemann, 1963; Marcus and Lengemann, 1962; Cramer, 1965; Schedl, Osbaldiston and Mills, 1968). The longer residence time of Ca^{2+} in the ileum compared to the duodenum, more than compensates for the greater rate of transit of Ca^{2+} across the duodenal epithelium. Marcus and Wasserman (1966) and Krawitt and Schedl (1968) have shown that the concentration of residual Ca^{2+} in the intestinal mucosa of the chick and rat was higher in the distal part of the small intestine than in the proximal part.

(2) Active transport of calcium

Many authors have tried to establish whether Ca^{2+} is transported "actively" i.e., using as a criterion the ability to transport against a concentration gradient or electrical gradients, or whether the transport process is susceptible to inhibitors of energy metabolism. In this way, Schachter and Rosen (1959), Dowdle, Schachter and Schenker (1960), Schachter, Kimberg and Schenker (1961), Kimberg, Schachter and Schenker (1961) and Cramer (1965) have found that the active Ca^{2+} transport is least active in the ileum and most active in the proximal segments of rats and dogs, rabbit and guinea

pigs under the influence of concentration gradient. Helbock, Forte *and* Saltman (1966) reported that Ca^{2+} transport against a concentration gradient was active in the presence of PO_4^{3-} but passive in its absence, but this was refuted by Walling and Rothman (1969) and Martin and DeLuca (1969a). Walling and Rothman (1968, and 1969), Martin and DeLuca (1969a), Adams, Wong and Norman (1970) and Adams and Norman (1970) showed that active transport could occur in the absence of a concentration gradient, but was dependent on the transmural potential. Krawitt and Schedl (1968) and Harrison and Harrison (1969) have determined that active transport occurs in all segments of rat small intestine. According to Harrison and Harrison (1969), this is not so for the rat colon. This active transport of Ca^{2+} from mucosal to serosal side of the intestine has been shown to be against a chemical gradient and metabolic energy is required as the transport is depressed by anaerobiosis, low temperature, sodium cyanide, iodoacetate, 2,4 DNP (Schachter and Rosen, 1959; Schachter et al., 1960a and b; Harrison and Harrison, 1960; Sallis and Holdsworth, 1962a). Wasserman, Kallfelz and Comar (1961) and Schachter (1963) reported that rat duodenum in vivo can transport Ca^{2+} against a chemical and an electrical gradient. Martin and DeLuca (1969b) in contrast to Hurwitz, Harrison and Harrison (1967), observed that reducing the Na^+ concentration in the medium in which the small intestine was incubated, depressed the active transport of Ca^{2+} against an electrochemical potential gradient. These results do not exclude the possibility of facilitated diffusion of Ca^{2+} into the cell from the lumen and blood. It is apparent that active transport occurs from the cell to the blood

and also the lumen. The overall process of Ca^{2+} transport from lumen to blood must therefore be an active process, i.e. the process of uptake into cell being possibly facilitated diffusion from the lumen, and transport out of the cell to blood, the active process.

CONDITIONS AFFECTING CALCIUM ABSORPTION IN THE SMALL INTESTINE

(1) The effect of pH

One factor favouring Ca^{2+} absorption in the small intestine may be that the proximal area of the intestine is at a lower pH value than the large intestine. Coates and Holdsworth (1961) measured the pH of the contents of the alimentary tract of chicks and found that in the gizzard the pH was about 2.6. The pH rapidly increased in the duodenum to 6.6 and then slowly increased to pH 7.9 at the distal end of the small intestine. Thus from these observations, it can be said that possibly the most proximal end of the small intestine would be the main area of absorption, as here the more insoluble Ca^{2+} will be made most soluble. The absorption of Ca^{2+} in the duodenum was observed to decrease in the rachitic chick, and increase slightly in the vitamin D_3 -replete chick as the pH of an injected Ca^{2+} solution was raised from about pH 2 to pH 6 (Wasserman, 1963). In the colon where the pH is lower and the digestion time is longer, there may be a larger amount of Ca^{2+} absorbed here than in the ileum and jejunum. However Harrison and Harrison (1969) found the rat colon absorbs less Ca^{2+} than ileum.

(2) The effect of calcium concentration

In the dog, with jejunal Thiry Villée fistulae, when there were low concentrations of Ca^{2+} , the rate of Ca^{2+} absorption was approximately proportional to Ca^{2+} concentration, but at higher concentrations the rate reached a maximum (Cramer and Dueck, 1962).

Wasserman (1963) showed that the total Ca^{2+} absorption from the intestine was nearly directly related to the concentration of Ca^{2+} in the chick intestine.

Bar and Hurwitz (1969a and b) studied the accumulation of Ca^{2+} and Ca^{2+} transport in laying fowl intestine in vitro. They found that the specific activity of $^{45}\text{Ca}^{2+}/^{40}\text{Ca}^{2+}$ of the accumulated Ca^{2+} was constant for a media containing 0.5 - 10.0mM- CaCl_2 . The intestinal accumulation of Ca^{2+} was linearly related to the activity of Ca^{2+} in the incubation medium.

In an experiment carried out by Zornitzer and Bronner (1970) using ligated loops in situ of the small intestine of rats with various solution of Ca^{2+} concentration (0.5 - 8 mg/ml) found that over this range of Ca^{2+} absorption was linear function of concentration at all ages (8-18 weeks).

(3) The effect of phosphate

If the $\text{Ca}^{2+}:\text{Pi}$ ratio in the diet of rats was decreased then Ca^{2+} absorption may be impaired due to the formation of insoluble salts (Irving, 1946). Cramer (1968) showed in dogs that when the Ca^{2+} concentration remained constant and either $\text{Ca}^{2+}:\text{Pi}$ or pH were altered, there was little change in Ca^{2+} absorption in vivo. Cramer (1968) also observed that the absorption of Ca^{2+} in the jejunum of the dog in situ was greater in the presence of Pi than in its absence. He found that when a constant amount of Ca^{2+} was perfused into the dog jejunum, increasing Pi/Ca^{2+} from 0.1 - 2.0 increased the rate of Ca^{2+} absorption. In this work, he chose Ca^{2+} , Pi and pH values for the perfusing fluid that did not form a precipitate.

Sernka and Borle (1969), in studying the intestinal contents of rats with varying amounts of Ca^{2+} in the diets, (below 3%) found that the concentration of free Ca^{2+} was less than the serum concentration for all segments of rat small intestine on all diets ($[\text{Ca}][\text{HPO}_4^{2-}] = 0.5\text{mM}^2$ in duodenum and 2.5mM^2 in ileum). The amount of bound Ca^{2+} was greater when Ca^{2+} was added to the diet and was the greatest in the ileal segment. They concluded that the complete absorption of Ca^{2+} from the duodenum may be followed by a small secretion of Ca^{2+} back into the lumen of the ileum. They remarked also that Ca^{2+} and PO_4^{3-} in the lumen exceeded the solubility product, but were kept in solution by possible binding to proteins.

(4) The effect of diet

Clark (1969a and b) demonstrated that provided the diet of rats contained sufficient Ca^{2+} to satisfy nutritional needs, a linear relationship was found between Ca^{2+} intake, the amount of Ca^{2+} absorbed and the serum Ca^{2+} level. He also reported in this article that Ca^{2+} absorption was unaffected by dietary PO_4^{3-} but serum Ca^{2+} was elevated when PO_4^{3-} intake was low. Further he reported that PO_4^{3-} absorption was linearly related to its intake when dietary Ca^{2+} was constant but inversely related to Ca^{2+} intake. Relationships were reported to have been found between absorbed to dietary and absorbed to serum ratio of $\text{Ca}^{2+}:\text{PO}_4^{3-}$ and $\text{Ca}^{2+}:\text{Mg}^{2+}$. By increasing the dietary $\text{Ca}^{2+}:\text{PO}_4^{3-}$ ratio, the serum Ca^{2+} level was raised while lowering the PO_4^{3-} and Mg^{2+} serum levels. Thus it was concluded that the $\text{Ca}^{2+}:\text{PO}_4^{3-}$ ratio in the serum was dependent on Ca^{2+} in the diet. It appeared that optimum Ca^{2+} and PO_4^{3-} in serum were required for bone formation

(Clark, 1968). The estimates obtained for daily Ca^{2+} and PO_4^{3-} requirements for young male rats on a diet consisting of adequate amounts of vitamin D and 20% excess of the required amount of essential minerals and other essential organic nutrients were 22 mg Ca^{2+} and 20 mg PO_4^{3-} per day for maximal growth and 43 mg Ca^{2+} and 34 mg PO_4^{3-} per day for maximal mineralization (Bernhart, Savini and Tomarelli, 1969). Relative to the Ca^{2+} requirement, more PO_4^{3-} was required for growth ($\text{Ca}^{2+}:\text{PO}_4^{3-}=1.1:1.0$) than for mineralization ($\text{Ca}^{2+}:\text{PO}_4^{3-}=1.0:1.4$).

In the hen, the electrochemical potential difference (EC-PD) in the duodenum and jejunum decreased as dietary Ca^{2+} decreased but always remained positive. However, the EC-Pd in chick in the upper intestine was positive with 1.11 and 0.71% Ca^{2+} diets but became negative in the entire intestine with 0.31% Ca^{2+} diet. Hurwitz and Bar (1969a) explained this by saying there was an uphill movement of Ca^{2+} into the cell. It was concluded that under conditions of Ca^{2+} restriction in chicks, active transport may assume an important role in the overall process of Ca^{2+} absorption (Hurwitz and Bar, 1969a). A net inflow of Ca^{2+} was observed in the ileum of hens fed the 3.94% Ca^{2+} diet but not with diets containing less Ca^{2+} .

In rats (Walling and Rothman, 1970) and laying hens (Hurwitz and Bar, 1969b), the percentage of Ca^{2+} absorption was increased when the animals were placed on Ca^{2+} depleted diets.

Zornitzer and Bronner (1970) found that 15 week old rats on a diet containing 0.05% Ca^{2+} and 0.2% PO_4^{3-} for 2-3 weeks were able to absorb Ca^{2+} as linear function of concentration, and the percentage of

absorption of Ca^{2+} was more than those animals on a diet containing 1.5% Ca^{2+} and 1.5% PO_4^{3-} , but lower than younger animals on this diet. These differences were not observed when animals were on a low Ca^{2+} diet for only 2-4 days. Animals that were raised on the low Ca^{2+} , PO_4^{3-} diet absorbed a higher percent of Ca^{2+} in the diet at 8 weeks of age than animals raised on high Ca^{2+} diet from weaning.

Walling and Rothman (1970) found that on analysis of the results of the in vitro experiments on rat intestine that the increased Ca^{2+} transport was due to affinity for Ca^{2+} by a protein molecule rather than an increase in the capacity of the transport process. They mentioned that this was analogous to the increase in sugar-carrier affinity in yeast, but added that rather than an actual change in carrier affinity, a second molecule could alter the carrier-substrate equilibrium. This then could produce a decrease in the observed affinity, if access to the carrier were rate limiting at low substrate concentrations, while the second molecule may increase the access to the substrate. They suggested this model was consistent with the changes in the Ca^{2+} transport system characteristics and the levels of CaBP under restricted Ca^{2+} dietary levels.

(5) The requirement for sodium in calcium transport

Calcium transport in vitro in the everted loop of distal small intestine of rats (Harrison and Harrison, 1963b) and in chicks (Hurwitz, Harrison and Harrison, 1967) and colon of rat (Harrison and Harrison, 1969) was found to be enhanced by a reduction in the concentration of Na^+ in the bathing medium.

More $^{45}\text{Ca}^{2+}$ was accumulated from Na^+ poor (5mM) medium than

from a Na^+ rich (125mM) medium by mucosal tissue (Bar and Hurwitz, 1969a). Neither ouabain nor phlorizin were found to inhibit transport accumulation of $^{45}\text{Ca}^{2+}$ from Na^+ poor medium. The oxygen consumption was lower in Na^+ poor medium than Na^+ rich medium. The accumulation of $^{45}\text{Ca}^{2+}$ was found to be linear regardless of Na^+ concentration (Bar and Hurwitz, 1969a).

In contrast, Martin and DeLuca (1969b) reported reduced Ca^{2+} transport in everted sacs of rat duodenum, when Na^+ was deleted from the medium. The reduced Ca^{2+} transport could not be accounted for by a change in the transmural electrical potential. Ca^{2+} was noted to move freely across the brush border of the epithelial cell even in the absence of Na^+ . Therefore Na^+ may enhance Ca^{2+} exchange at the basal membrane of these cells.

The Ca^{2+} transport system in vitro with everted rat intestine was investigated in the presence of ethacrynic acid by Birge, Gilbert and Avioli (1972), who found this substance inhibited Ca^{2+} transport. A phosphatase dependent on Na^+ and Ca^{2+} was also inhibited, while ouabain was without an effect. This phosphatase was associated with the basal and lateral membranes of the mucosal cells.

(6) The effect of the formation of insoluble salts of calcium

There have been suggestions that the presence of phytates from plants may interfere with Ca^{2+} absorption by the formation of insoluble salts (Nicolaysen and Eeg-Larsen, 1953; Irving, 1957; Wasserman, 1960; Davis, 1963). Wasserman (1962) reported that PO_4^{3-} and phytate equally depressed $^{45}\text{Ca}^{2+}$ absorption in chicks and to overcome this it was proposed that Ca^{2+} in the diet be increased to maintain the

$\text{Ca}^{2+}:\text{PO}_4^{3-}$ ratio at a proper level or to increase the amount of vitamin D in the diet.

The solubilization of sparingly soluble salts would be of considerable physiological importance, as most Ca^{2+} in the normal birds diet is in this form. In the lower portion of the small intestine, Ca^{2+} may be solubilized by the introduction of bile, thus presenting Ca^{2+} in a diffusible form in the higher pH ranges of the small intestine. At the same time, unabsorbed fats may form insoluble Ca^{2+} soaps and thus interfere with absorption of Ca^{2+} if the bile duct is obstructed (Irving, 1957). Webling and Holdsworth (1965) have shown that bile from rachitic or normal chicks caused an immediate increase in the intestinal absorption of soluble Ca^{2+} in rachitic and vitamin D_3 -treated chicks, as tested in vivo by intestinal-loops and by oral dosing methods. They also noted that the effect was due to the taurine conjugated bile acids present in the bile and was independent of the action of vitamin D. The effect of bile on Ca^{2+} absorption was shown to be independent of the effect on bile in absorption of fat soluble vitamins. Chick bile and isolated bile salts were also shown to increase the water solubility and absorption of Ca^{2+} present as sparingly soluble salts, e.g. CaHPO_4 and phytate at pH values 6-8, conditions similar to those in the small intestine. Using rachitic chicks or normal rats, Webling and Holdsworth (1966) showed a decrease in Ca^{2+} absorption from CaHPO_4 when the bile ducts were tied, but was restored by giving bile salts with the Ca^{2+} salt. The enhancement of Ca^{2+} absorption by bile was observed in all regions of the small intestine of the chick. Bile salts that are not present

in chick bile, e.g. glycine conjugates and bile alcohol sulphates as well as the synthetic compound sodium lauryl sulphate were also shown to enhance Ca^{2+} absorption (Webling and Holdsworth, 1966). In an attempt to explain the action of bile on soluble Ca^{2+} salts, the authors correlated the increased absorption due to bile salts with the increased lipid solubility of Ca^{2+} in the presence of bile, and suggested that this lipid-soluble Ca^{2+} could pass across membranes more easily than Ca^{2+} and insoluble Ca^{2+} salts.

Fakambi, Flanzy and Francois (1969) have suggested that saturated fatty acid in combination with Ca^{2+} helps to liberate phosphorus for absorption. This is as a result of calcium phosphate being converted to insoluble Ca^{2+} soaps, thus liberating the phosphate for absorption. There has been a suggestion put forward by Munday, Parsons and York (1970) that Ca^{2+} transport and long chain fatty acid transport may be interrelated at both brush borders and at the basal and lateral membranes of the intestinal mucosa epithelial cells, as there was a large increase in palmitic acid uptake by rat jejunum slices in the presence of Ca^{2+} in the incubation medium at 0°C and 37°C . Williams, Rose, Morrow, Sloan and Barness (1970) reported a similar correlation between Ca^{2+} and fatty acid absorption in infants at 4 to 11 days of life. The loss of Ca^{2+} in excretion was also related to fatty acid composition of milk, e.g. low stearate and palmitate, high oleate promoted retention of Ca^{2+} .

(7) The effect of amino acids, sugars and other metabolites on calcium absorption.

It has been suggested that amino acids which are also

absorbed in the small intestine may help to increase the solubility of phosphates and carbonates of Ca^{2+} (Lehmann and Pollak, 1942). This was found to be true in the absorption of Ca^{2+} in rat intestine in the presence of lysine (Wasserman, Comar and Nold, 1956), but was not observed in the rachitic chick after an oral administration of lysine and arginine (Wasserman, Comar, Schooley and Lengemann, 1957) and was independent of tyrosine uptake (Harrison and Harrison, 1963a).

It was observed that feeding large amounts of citrate in the diet prevented rickets in rats but this was not the case in other species (Nicolaysen and Eeg-Larsen, 1953). Pileggi, DeLuca, Cramer and Steenbock (1956) suggested that dietary citrate cured rickets merely by complexing with Ca^{2+} , which makes phytate phosphorus unavailable. The phytate then being easily hydrolysed by the phytate enzyme making phosphorus available. The phosphorus produces a cure of rickets which was duplicated by feeding an equivalent amount of inorganic phosphorus. Schrier and Schnepf (1956) studied the effect of orally administered citrate and observed that it increased Ca^{2+} absorption in both rachitic and vitamin D treated rats. Wasserman (1962) found that citrate had no effect on Ca^{2+} absorption in chick small intestine. Chelating agents (EDTA) and citrate which reduce the Ca^{2+} activity were found to reduce the accumulation to less extent than expected suggesting that the complex was accumulated (Bar and Hurwitz, 1969a).

In an experiment on the effects of glucose, lysine and lactose on Ca^{2+} absorption on the small intestine of rats, Lengemann and Comar (1961) found that these substances significantly increased absorption in the ileum but only lysine increased absorption in the duodenum. It was shown that lactose increased Ca^{2+} absorption when fed along with a normal diet (Fournier, 1954). Lactose has been shown

by various workers (Wasserman et al, 1957; Lengemann, Wasserman and Comar, 1959; Wasserman and Lengemann, 1960) to have no influence on pH of the intestine or on the role of bacteria, but it did on Ca^{2+} absorption and this effect was decreased by the simultaneous presence of EDTA. Thus lactose appears to influence the translocation of ionized Ca into plasma. From experiments by Fischer-Moiruddin and Lee (1958), and later by Fournier, Susbielle and Bescal-Liversac (1959) the role of lactose in intestinal Ca^{2+} absorption was thought to be due to an increase in the surface area available for absorption. Lactose was shown to stimulate caecal development and to a lesser extent influence the development of the small and large intestine. However, when the caecum was removed, the effect of lactose upon Ca^{2+} absorption was not altered. It has been shown that lactose and Ca^{2+} have to be administered in the diet at the same time, before the lactose effect can be observed (Lengemann, 1959). Vaughan and Filur (1960) have shown that other carbohydrates such as glucose, galactose, fructose, sorbital and D-xylose increase Ca^{2+} absorption in the ligated ileum. Lengemann and Comar (1961) confirmed that glucose increased Ca^{2+} absorption in the ileum, and suggested that these carbohydrates helped absorption by maintaining Ca^{2+} in a soluble form. Bar and Hurwitz (1969a) reported that the stimulation of Ca^{2+} uptake by intestine in vitro was greater with fructose than glucose. In a study of Ca^{2+} absorption by humans, Pansu and Chapuy (1970) mentioned that glucose had no effect on Ca^{2+} absorption, but lactose and xylose stimulated Ca^{2+} absorption.

(8) Growing and Pregnant animals

In growing or pregnant animals, there was a larger requirement for Ca^{2+} which was required for bone formation and during lactation. To compensate for the increased requirement, Ca^{2+} absorption was increased as shown by a study of calves and cows (Lengemann, Comar and Wasserman, 1967), in humans (Bedford, Harrison, Raymond and Sutton, 1960; Spencer, Li, Samachson and Laszlo, 1960; Straub, Kahn and Tellis, 1961; Shenolibar, 1970), in ewes (Braithwaite, Glascock and Riazuddin, 1970) and in lactating rats (Kostial, Gruden and Durakovic, 1969).

A study of the effect of abnormal maternal plasma Ca^{2+} levels on the offspring of rats by Fairney (1969) and Fairney and Weir (1970), revealed that the plasma levels of Ca^{2+} of the maternal rat had a severe effect on the growth and plasma of Ca^{2+} levels in their offspring. Rats on low Ca^{2+} diets, or with hypocalcemia, following PTX produced smaller litters than normal. The offspring were born with low birth weights and grew poorly compared to the controls. The levels of the offspring plasma Ca^{2+} rose to normal by the sixth day after birth. The growth defect of the offspring of the hypocalcemic rats was reversed if offspring were reared by normal rats during the first 19-24 hours of life.

(9) The effect of germ free conditions

A study on rats that were germ free was carried out by Reddy, Pleasants and Wostman (1969) in order to measure the effect of intestinal flora on Ca^{2+} , PO_4^{3-} and Mg^{2+} metabolism. They noted that neither the germ free status nor diet used, appreciably influence

serum Ca^{2+} , PO_4^{3-} and Mg^{2+} levels. Both germ free conditions and feeding of liquid diet increased apparent absorption between germ free and conventional animals, but feeding of liquid diet alone resulted in a higher absorption. They concluded that the presence of microflora in the gut influenced the metabolism of Ca^{2+} , PO_4^{3-} and Mg^{2+} .

In conclusion, Ca^{2+} absorption (i) is linear to Ca^{2+} concentration and is not saturable, (ii) depends on age and previous dietary history, (iii) influenced by substances that increase or decrease Ca^{2+} solubility and (iv) influenced to a lesser extent by a number of other factors, e.g. sugars, amino acids, Na^+ , germ free conditions and pH.

ABSORPTION OF PHOSPHATE FROM THE INTESTINE

Numerous studies have shown that increasing the $\text{Ca}^{2+}:\text{PO}_4^{3-}$ ratio in the diet may interfere with the absorption of phosphate.

Phosphate absorption according to Hurwitz and Bar (1970) occurs mostly in the upper jejunum in 3 week old chicks. The uptake of Ca^{2+} by everted intestinal sacs from rat was not dependent on phosphate in the diet or medium, but the reverse appears to be true (Harrison and Harrison, 1961; Walling and Rothman, 1969). A different view was expressed by Kowarski and Schachter (1969); phosphate transport was maximal in the rat jejunum and relatively independent of Ca^{2+} in the vitro bathing medium. According to Helbock et al (1966), the uptake of phosphate ions in vitro was an active process, as inhibitors of oxidative phosphorylation and glycolysis inhibited the process. In the rat, the duodenal Ca^{2+} transport was maximal while

PO_4^{3-} transport was maximal in the ileum in vitro. It is not known whether there is a mechanism for the regulation of PO_4^{3-} absorption from the intestine. The difficulty arises because it has not been possible to differentiate between a primary effect on PO_4^{3-} or a secondary effect due to an effect on Ca^{2+} absorption.

The effect of Parathyroid Hormone (PTH) and vitamin D on intestinal absorption will be discussed in later sections.

THE ROLE OF VITAMIN D IN NUTRITION

Rickets is now recognized as a deficiency of vitamin D. In addition to vitamin D deficiency, it is possible to produce rickets by low PO_4^{3-} or low Ca^{2+} diet and very high PO_4^{3-} diet. Rats on a vitamin D deficient diet containing 1-2% Ca^{2+} developed rickets when the PO_4^{3-} level was low but did not when the PO_4^{3-} level was high (Krawitt and Kunin, 1971). Rickets is characterized by a failure of the mineralization process to keep pace with the formation of the organic matrix of bone. This results in a widening of the epiphyseal plate, which is unable to support the weight of the animal. Thus the bones become malformed, bowed and wasted. Rickets has been reviewed by Loomis (1970).

Vitamin D will cure the disease. It has been postulated that there are 3 sites of action, e.g. intestine, bone and kidney. This will be dealt with in the following sections.

(1) The effect of vitamin D on calcium absorption

The topic of the effect of vitamin D on the intestinal Ca^{2+} absorption has been recently reviewed by Rasmussen and DeLuca (1963), DeLuca (1967), Norman (1968), Wasserman (1968), Adams (1970)

and DeLuca (1971).

It was originally thought that in the vitamin D- deficient animal there was an increased output of Ca^{2+} and PO_4^{3-} in the faeces. This was disproved by Nicolaysen and co-workers who clearly demonstrated that the effect of vitamin D was to promote Ca^{2+} and phosphate absorption (Nicolaysen and Eeg-Larsen, 1953). This led to the conclusion that the primary effect of vitamin D was upon Ca^{2+} absorption while increased phosphate absorption was a secondary phenomenon. Steenbock and Black (1924) presented evidence that vitamin D action was upon Ca^{2+} absorption and this was substantiated by thorough studies utilizing $^{45}\text{Ca}^{2+}$ in rats (Carlsson, 1951; Lindquist, 1952) and in chicks (Migicovsky and Nielson, 1951; Keane, Collins and Gillis, 1956; Coates and Holdsworth, 1961; Wasserman and Taylor, 1962; Wasserman, 1962). Many workers have approached the problem of whether the vitamin D-mediated increase of Ca^{2+} transport is an active or passive process by either (a) utilizing everted intestinal sacs, intestinal slices, or isolated villi in vitro and studying the distribution of $^{45}\text{Ca}^{2+}$ in the mucosal and or serosal compartments or (b) by studying $^{45}\text{Ca}^{2+}$ flux in vivo with ligated intestinal loops. One can criticize the in vitro everted sac technique because, in the process of eversion, the tight junction between adjacent cells may be disrupted. Also the fuzzy layer above the microvilli may be disturbed and the blood flow has been eliminated. With many of the methods used the total volumes of the serosal and mucosal fluids were very different, 0.5 - 1.0 ml and 2.5 - 5.0 ml respectively, which may lead to errors in membrane absorption and

adsorption. The in vitro experiments are carried out in unphysiological conditions and therefore Ca^{2+} is being transported in a rather artificial state across the membranes.

Sallis and Holdsworth (1962a) after studying the effect of vitamin D in vivo and in vitro Ca^{2+} transport concluded that the Ca^{2+} transport systems were markedly different as similar amounts of Ca^{2+} were absorbed along the entire small intestine of chicks in vivo, while the maximum transport in the everted sac occurred in the distal third of the small intestine. Holdsworth (1965) also reported a difference in the in vivo and in vitro Ca^{2+} transport system. They both noted there was a trend to accumulate more Ca^{2+} in normal and vitamin D_3 -treated mucosal cells than rachitic cells in vitro, whereas the situation in vivo was the reverse. A similar comparison of in vivo and in vitro Ca^{2+} transport in rat was carried out by Urban and Schedl (1969b). The in vivo studies were made by steady state intraluminal recirculation through the duodenum and ileum of solutions of Ca^{2+} at concentrations below 0.4mM and above 3.4mM serum ionised Ca. The in vitro studies were carried out in the everted duodenal sacs at 0.4mM- Ca^{2+} solution before and after the in vivo studies. There was a net Ca^{2+} absorption in both duodenum and ileum against a concentration gradient in vivo. Absorption and lumen to plasma flux were always greater in the duodenum. Vitamin D was found to increase the absorption as well as the lumen to plasma flux in vivo only in the duodenum and only at 3.4mM- Ca^{2+} . The everted duodenal sacs showed a highly significant enhancement of the serosal to mucosal ratio of $^{45}\text{Ca}^{2+}$ by vitamin D even at 0.4mM- Ca^{2+} .

They concluded that in vivo and in vitro studies were not strictly comparable because of the fundamental physiological differences. Vitamin D was suggested to increase Ca^{2+} transport only against a limited concentration gradient in vivo and there may be 2 mechanisms, one independent of and the other dependent on vitamin D for stimulation.

Schachter et al (1960a) stated that vitamin D increased the level of active transport mechanism at the basal membrane. However from measurements of unidirectional fluxes of Ca^{2+} from lumen to plasma and plasma to lumen across the chick duodenum in vivo made by Wasserman and Kallfelz (1962), it was concluded that vitamin D did not operate through unidirectional orientated active transport, but affected the permeability of mucosa cell to Ca^{2+} in both directions. This was confirmed by Wasserman, Taylor and Kallfelz (1966). Olson (1969) observed that in perfusion experiments with vitamin D-deficient rat's small intestine, that the lumen to plasma flux was half the value for the intestine in vivo from a vitamin D-supplemented rat. This difference was reported to be maintained over a period of 4 hours. The accumulation of Ca^{2+} in a vitamin-D replete animal was shown to be more rapid than that in the rachitic animal. The level of Ca^{2+} in vitamin D tissue also fell more quickly in the vitamin tissue till it equalled or was less than the rachitic tissue. The vitamin D mucosa always contained more luminal Ca^{2+} and was in rapid equilibrium with the luminal Ca^{2+} (Wasserman, 1963; Wasserman, 1968). However, the Ca^{2+} from the lumen in the mucosal cell did not exchange with the residual Ca^{2+} within the cell. During this preabsorptive state, the cellular concentration was 1mM and rose

to 3-5mM in the absorptive phase. Neville and Holdsworth (1968) showed that administration of vitamin D₃ 16 hours before the experiment increased the Ca²⁺ absorption of the chick ileum as compared to rachitic chicks. They also found after 1 hour that the content of the rachitic mucosal cells contained more Ca²⁺ than vitamin-replete chicks. These results indicated an increased passage of Ca²⁺ to plasma and reduced efflux from the vitamin D-replete cells.

Urban and Schedl (1969b) reported that the concentration of Ca²⁺ in the tissue of the ileum was higher than that in the duodenum. When the luminal Ca²⁺ concentration was below the serum level of Ca²⁺, then vitamin D had no effect on the Ca²⁺ tissue concentration (Urban and Schedl, 1970), but when the luminal Ca²⁺ concentration was above the serum Ca²⁺ level (3.4mM-Ca²⁺) then the tissue concentration of Ca²⁺ decreased and the specific activity of the mucosal fraction decreased in both segments of the vitamin D-replete rat intestine. In other words, vitamin increased the entry and exit fluxes at the basal poles of the cells, there being only a minimal effect on the entry flux at the luminal surface. No vitamin D effect was noted for the underlying tissue. Vitamin D had no effect on the endogenous ⁴⁰Ca²⁺ of the perfused tissue, nor was the ⁴⁰Ca²⁺ content of the perfused tissue influenced. These findings demonstrate that the major site of vitamin D action was on the mucosal release step in the transport of Ca²⁺ from lumen to blood.

Haskim and Clark (1969) showed that the mucosal cells of the small intestine obtained from rats deprived of vitamin D or given excessive amounts of the vitamin, accumulated significantly more Ca²⁺ than did cells from

control animals, while the mucosal cells from vitamin D-deficient rats released less Ca^{2+} than did cells from normal or hypervitaminotic D animals. Their in vivo studies showed that the transfer of $^{45}\text{Ca}^{2+}$ from the intestine to the blood was delayed in vitamin D deficiency, but was accelerated in hypervitaminosis D.

Adams, Wong and Norman (1969 and 1970) reported that one of the major effects of an in vivo administration of vitamin D to a vitamin D-deficient chick was to increase by 2-3 fold the lumen to plasma flux of Ca^{2+} in the intestine.

Haskim and Clark (1969) reported that actinomycin D had no effect on Ca^{2+} uptake by the mucosal cells of rat duodenum. $^{45}\text{Ca}^{2+}$ was observed in the proximal and distal parts of the rat intestinal mucosa at 5 and 30 minutes after an oral administration (Moruichi, Watanabe and Hosoya, 1970). Serum $^{45}\text{Ca}^{2+}$ concentration at 30 minutes was increased significantly in the vitamin D_3 treated rat, and this vitamin D_3 effect was inhibited by actinomycin D. These actions were not observed at 5 minutes. $^{45}\text{Ca}^{2+}$ in the proximal parts at 5 minutes was reduced with vitamin D_3 , while actinomycin D did not appear to influence vitamin D action at this time. $^{45}\text{Ca}^{2+}$ uptake in the distal part of the intestine at 30 minutes was enhanced by vitamin D_3 , and this action was partially inhibited by actinomycin. The subcellular distribution of $^{45}\text{Ca}^{2+}$ was studied in the duodenal mucosa. Nuclei and cell debris (brush borders) of the duodenum were found to contain relative larger amounts of $^{45}\text{Ca}^{2+}$ at 5 minutes after administration than at 30 minutes. Less Ca^{2+} was found in organelles 30 minutes after an oral dose when the rats had received vitamin D

(Moriuchi, Watanabe and Hosoya, 1970). Actinomycin D inhibited the action of vitamin D only on the microsome and supernatant fractions by decreasing the amount of $^{45}\text{Ca}^{2+}$ in these fractions. To explain the actions of actinomycin they suggested that Ca^{2+} transport could be carried out in two ways : (i) by a membrane permeable system which was actinomycin D insensitive and (ii) by a system involving Ca^{2+} carrier protein that was actinomycin sensitive. The second being the more likely at 30 minutes.

Small electron dense granules and the autographic label of Ca^{2+} were shown to be primarily present in the microvilli and mitochondria of mucosal cells of normal, rachitic and vitamin D-treated rat small intestine (Sampson, Matthews, Martin and Kunin, 1970). These granules were sparse in mitochondria from rachitic rats intestinal sections, while they were regularly displayed in those from normal rat gut sections. The mitochondria observed in sections from vitamin D-treated rat intestine showed numerous granules. These granules were limited to the microvillus region in rachitic animals and increased significantly in the mitochondria following treatment with vitamin D. This indicates that a large amount of Ca^{2+} is absorbed across the cell membrane and was then found within the microvilli. Vitamin D, thus was necessary for the mobilization of Ca^{2+} from the microvilli. The mobilized Ca^{2+} enters the mitochondria or passes through the cell to the adjacent blood vessel.

A number of aspects of the effect of vitamin D on in vitro Ca^{2+} translocation have been investigated. Some of these aspects were

(1) the effect on Ca^{2+} translocation, (2) the effect of vitamin D under conditions where oxidative phosphorylation had been inhibited, (3) the effect of the vitamin on permeability of mucosal surface, (4) the unidirectional flux, (5) the effect under glycolysis and (6) the effect of Na^+ concentration on Ca^{2+} translocation.

Schachter and co-workers, utilizing the everted sac technique, were able to conclude that the Ca^{2+} transport from mucosa to serosa in rabbit, rat and guinea pig small intestine was an active process dependent upon vitamin D, and was primarily limited to Ca^{2+} as there was no significant transfer of Ba^{2+} , Sr^{2+} , or K^+ (Schachter and Rosen, 1959; Dowdle et al, 1960; Schachter et al, 1960a and b).

The flux from mucosa-serosa of Ca^{2+} in the everted sac was found to increase in vitamin D-treated rats as compared with those that were vitamin D-deficient (Harrison and Harrison, 1960). Sallis and Holdsworth (1962a) using chick small intestine also obtained similar results.

In studies with chick duodenum, Wasserman (1962) established that $^{47}\text{Ca}^{2+}$ uptake by mucosa of everted gut tissue was enhanced by vitamin D and also the rapid uptake of Ca^{2+} from a ligated duodenal loop of a chick was dependent upon the vitamin D status of the animal.

Ca^{2+} transport in the rat duodenum (Martin and DeLuca, 1969a; Walling and Rothman, 1969) and in chick ileum (Adams, Wong and Norman, 1969; Adams and Norman, 1970) in vitro has been reported to be an active process against an electrochemical potential gradient. This process (Martin and DeLuca, 1969a; Adams et al, 1969, 1970) was an energy dependent, cation oriented reaction, which was markedly enhanced by

vitamin D. The Ca^{2+} transport occurred in the absence of a concentration gradient (Walling and Rothman, 1969) and in the absence of phosphate (Martin and DeLuca, 1969a), and diminished with age (Walling and Rothman, 1969).

Walling and Rothman (1969) studied the effect of 100 fold range in concentration of Ca^{2+} (0.125-12.5 mM) on the $\text{M} \rightarrow \text{S}$ flux and discovered it to be saturated at about 4 mM, while the $\text{S} \rightarrow \text{M}$ flux increased proportionately with Ca^{2+} concentrations. The $\text{M} \rightarrow \text{S}$ flux was approximately doubled in rats fed low Ca^{2+} diet, while $\text{S} \rightarrow \text{M}$ flux was unchanged, which was inferred to mean an increase in active transport capacity. This saturation of low Ca^{2+} diet enhancement and vitamin D dependence of the $\text{M} \rightarrow \text{S}$ flux process was suggested to mean a carrier mediated Ca^{2+} transport.

The Ca^{2+} uptake process of mucosal cells was also saturable with Ca^{2+} (2.0 mM) and showed a requirement for oxygen in this in vitro incubation system (Martin and DeLuca, 1969a). Intestinal mucosa from vitamin D-deficient rats behaved similarly, but demonstrated a reduced rate of Ca^{2+} uptake. The rate limiting process in vitamin D-deficiency appears to be the initial transfer of Ca^{2+} across the brush borders surface into the columnar epithelial cells.

Harrison and Harrison (1960) found that when the intestinal sac were incubated under nitrogen, at reduced temperature or in the presence of cyanide ($5 \times 10^{-4} \text{M}$) to inhibit oxidative phosphorylation, the vitamin D effect was not inhibited under these conditions. Schachter et al (1961) utilizing intestinal slices and everted intestinal sacs, noted that oxygen and vitamin D were required for

accumulation of $^{45}\text{Ca}^{2+}$ in the cells. All parts of the small intestine showed an increase in active transport of Ca^{2+} due to vitamin D, of 74% in the presence of O_2 , but only 7% in the presence of N_2 . This accumulation was also depressed by cyanide, azide, and 2,4 dinitrophenol (2,4 DNP). The transportable sugars (D-glucose) decreased the oxygen dependence of $^{45}\text{Ca}^{2+}$ uptake. Sallis and Holdsworth (1962a), utilizing sacs of chicks, concluded on the basis of inhibitors studies that vitamin D-mediated Ca^{2+} transport was energy dependent, and the energy could be derived from glycolysis. Anaerobic conditions, however, were found not to inhibit $^{45}\text{Ca}^{2+}$ exchange, but inhibited active transport. The uptake of Ca^{2+} by mucosa of everted gut was enhanced by vitamin D, and the uptake by the tissues was shown by Wasserman (1963) not to be appreciably affected by NaCN, anaerobic conditions or 2,4, DNP. The entrance of Ca^{2+} into the tissue was therefore not dependent on oxidative metabolism. This uptake into mucosal cell was independent of oxidative metabolism according to Brock and Schedl (1969). In an experiment similar to those of Harrison and Harrison (1965), Schachter, Kowarski, Finkelstein and Ma (1966) using everted intestine of rats, separated the mucosal tissue from the underlying muscle coats after incubation of everted sacs in $^{45}\text{Ca}^{2+}$ labelled medium. The uptake of $^{45}\text{Ca}^{2+}$ from the mucosal medium was only partially inhibited by anaerobiosis and 2,4 DNP. They concluded that the process of entry into the cell was one of facilitated diffusion. Their results supported the observations made by Wasserman (1963). Haskim and Clark (1969) have reported that mucosal cells scraped from rat duodenum took up more Ca^{2+} by a passive process at

0°C than at 38°C. When glucose was omitted from the incubation medium, there was 50% decrease in uptake. This effect was not noticed under nitrogen. In the presence of cyanide ($8.5 \times 10^{-5} \text{M}$) or arsenate (10^{-2}M), uptake was double that of the controls. This could mean that when mucosal cells were scraped from their support, Ca^{2+} entered by the freely permeable membrane on the non-luminal surface, or that these cells were not intact. Adams et al (1969, 1970) found that low temperature and inhibitors of sulphydryl groups inhibited $\text{Ca}^{2+} \text{ M} \rightarrow \text{S}$ flux in vitamin-treated chicks, but did not affect the vitamin D-deficient chick flux in vitro in ileum.

In a further study in this regard, Harrison and Harrison (1970) measured the diffusibility of Ca^{2+} across the wall of rat small intestine in vitro everted intestinal sacs, incubated under conditions to eliminate energy linked active transport of Ca^{2+} e.g. 22°C under anaerobic conditions. In this system vitamin D-treatment of the animals was reported to increase the in vitro mucosal to serosal transfer of Ca^{2+} . The pre-treatment of the everted duodenal loop with dibutyryl CAMP and theophylline in vitro, caused a further increase in mucosal to serosal transfer of Ca^{2+} in preparations from vitamin D treated rats, but there was no effect in those from vitamin D-deficient rats. The same concentration of theophylline alone did not alter Ca^{2+} transfer. The prior administration of dibutyryl CAMP alone to the rats before removal of the intestine, also caused an increase in the in vitro diffusibility of Ca^{2+} across the intestine of vitamin D-treated rats but not of vitamin D-deficient rats. This is in contrast to that reported by Neville and Holdsworth (1969) who showed an

increase in Ca^{2+} translocation due to DCAMP in vitamin D-deficient ileum and no effect on vitamin D-treated ileum in vivo and in vitro experiments. Neville and Holdsworth (1969) found a slight increase of adenyl cyclase activity after treatment with vitamin D, but whether cyclic AMP was a second messenger involved in the effect of vitamin D was not known.

In an early study, Harrison and Harrison (1960) suggested that, as vitamin D affected the permeability of the mucosal surface to Ca^{2+} , and if this were the limiting step, then this would also limit the energy-dependent active Ca^{2+} transport, as the availability of Ca^{2+} would be diminished. In a further report, they gained support for the concept when they incubated everted intestinal sacs, from which the mucosal epithelium had been removed, and normal sacs in the absence and presence of 0.5 mM-N-ethyl maleimide (N.E.M.), to inhibit active transport at 37°C or 5°C (Harrison and Harrison, 1965). All the in vitro results obtained were in accordance with the concept that vitamin D increases the uptake step of Ca^{2+} by the mucosal cell by reducing the permeability barrier. This is in agreement with studies of filipin on the mucosal cell membrane permeability by Adams and co-workers (1969, 1970). There was no permeability difference between the +D and -D system of ileum as judged by [^{14}C]thiourea and $^3\text{H}_2\text{O}$ fluxes in the in vitro, and filipin had no effect on these fluxes (Adams and Norman, 1970 ; Adams et al, 1969, 1970). Filipin, a polyene antibiotic, when present in the mucosal solution caused a two to threefold increase in Ca^{2+} flux only with vitamin D-deficient ileum (Adams et al, 1969, 1970). The incubation of filipin in vitro with

vitamin D-deficient ileal tissue mimicked many aspects of dietary vitamin D administration in that (1) induced translocation of Ca^{2+} against an electrochemical gradient, (2) confers the property of cold sensitivity on the increased mucosal to serosal flux, (3) confers the sensitivity to N.E.M. on this increased M \rightarrow S flux, (4) interacts with the membrane bound carrier on the microvilli side of the cell to effect an increase uptake of Ca^{2+} into the cell and (5) binds more rapidly to intact ileal segments obtained from vitamin D-deficient compared to vitamin D-replete chicks. Since there was no direct reaction between Ca^{2+} and filipin, filipin may effect a structural reorganization of the vitamin D-deficient microvilli membrane so the Ca^{2+} transport process becomes "active". This filipin effect was remarkably similar to that direct actinomycin D-sensitive effect brought about by dietary vitamin D administration. The magnitude of filipin-mediated increase in Ca^{2+} flux was inversely proportional to the time after vitamin D administration in vivo to rachitic chicks, so that 60 hours after vitamin D there was no filipin effect. Adams et al (1970) and Wong, Adams, Roberts and Norman (1970) showed that filipin was found to bind to a greater extent, and at a faster rate, to vitamin D-deficient than vitamin D-repleted chick intestine. From electron micrographic studies, it could be seen that prolonged exposure to filipin in vitro caused gross morphological changes in the microvillar region of the ileal segment of vitamin D-deficient chicks, but were not seen in repleted chicks after only short periods of time of 20 to 40 minutes, at which time no morphological changes could be detected.

Rasmussen, Waldorf, Dziewiatkowski and DeLuca (1963)

observed that in a preparation of isolated rat villi (i.e. the serosal barrier removed), 95% of the external Ca^{2+} (0.1mM) entered in 10 minutes at 4°C , while only 20% entered at 33°C . When the villi were loaded with Ca^{2+} at 4°C and the temperature was raised to 33°C , there was a rapid off-loading of the accumulated ions. Inhibition of oxidative phosphorylation, electron transport and substrate oxidation were able to prevent the uptake at 4°C . The vitamin D-replete rat villi were able to accumulate and release Ca^{2+} to about the same extent as those from vitamin D-deficient rats. Similar results were obtained with chick mucosal cells by Holdsworth (1965). Brock and Schedl (1969) reported that isolated villi from duodenum of vitamin D-deficient rats showed that Ca^{2+} uptake was significantly greater than from the villi of the vitamin D-treated animals. While villi from the ileal region showed no influence of vitamin D, the Ca^{2+} content of the tissue of the vitamin D-depleted villi was significantly greater than in the repleted rats. To explain these results, they postulated that Ca^{2+} was taken up by the epithelial cells of the isolated villi and was released through the basement membrane into the solution. Thus vitamin D may promote Ca^{2+} release from the basal membrane of the epithelial cell. These experiments were in contrast to experiments where the underlying muscle was not removed prior to incubation, where vitamin D was found to increase the uptake of Ca^{2+} into the mucosa (Wasserman, 1963; Holdsworth, 1965; Schachter et al, 1966).

There is general agreement that vitamin D does enhance Ca^{2+} translocation and that Ca^{2+} translocation is dependent upon oxidative metabolism for the active transport process of crossing the intestine,

but there have been a number of theories proposed on ways in which vitamin D affects Ca^{2+} transport. Vitamin D increases the rate of Ca^{2+} transfer from lumen to plasma, but whether this is a direct effect on a Ca^{2+} pump, on the permeability of the membranes, or is induced as consequence of its effect elsewhere in the cell is still unsettled. An early theory was that of Schachter and co-workers (1961). They suggested that two steps were involved in the translocation of Ca^{2+} . The first of these was the mucosal uptake and the second step was the transfer to the serosal fluid in vitro or to the blood stream in vivo. The second step was dependent on a metabolizable substrate in the in vitro system. Vitamin D was required for each of the steps and only increased the unidirectional fluxes toward the serosa in the direction of absorption.

Harrison and Harrison (1965) proposed that vitamin D acted by increasing the membrane permeability of the mucosal membrane to Ca^{2+} . Thus vitamin D would increase the uptake step of Ca^{2+} by the mucosal cell by reducing the permeability barrier.

In the model proposed by Holdsworth (1965) for Ca^{2+} transport, the two faces of a sheet of mucosal cells were considered to have two properties, (a) a unidirectional Ca^{2+} pump operated by metabolic energy pumping Ca^{2+} out of the cell, (b) a diffusion leak into and out of the cell. Here it was proposed that vitamin D inhibited a metabolically operated pump that returns Ca^{2+} from the mucosal cell to the intestinal lumen.

Wasserman (1968) suggested that transport from lumen to mucosal cell was neither dependent upon oxidative metabolism, nor

readily saturated by Ca^{2+} ion, but was dependent upon vitamin D. He proposed that the mucosal Ca^{2+} pool was proportionate to the luminal Ca^{2+} and that the size of the pool was increased by vitamin D. This Ca^{2+} in transit was not totally exchangeable with the structural cell Ca^{2+} and was probably membrane bound or fixed to anionic sites. The flux from mucosal cell to plasma was thought to be by a pump located on or near basal and/or lateral surfaces. This pump was functionally significant at low intraluminal levels of Ca^{2+} , was energy dependent and directly or indirectly dependent on vitamin D. At higher levels of intraluminal Ca^{2+} there may be passive diffusion, which also may be dependent directly or indirectly on vitamin D. The flux from plasma to mucosal cell was stated not to be affected by vitamin D, but was possibly energy dependent and temperature sensitive. Wasserman proposed that the flux from the mucosal cell to the lumen was vitamin D dependent and influenced by the relative concentration of Ca^{2+} in the lumen and plasma. The findings of Haskim and Clark (1969) support the hypothesis that vitamin D was involved in the release of Ca^{2+} rather than in its uptake by mucosal cells. DeLuca (1969b) proposed that Ca^{2+} was transferred across the brush border of the mucosal cell by means of an ATPase. In the cytoplasm, Ca^{2+} then became associated with the calcium binding protein which formed as a result of vitamin D_3 stimulation of protein synthesis. At the serosal border of these cells, Ca^{2+} became detached from the binding protein and calcium was exchanged for Na^+ .

(2) The effect of vitamin D upon the skeleton

This topic has been reviewed by Nicolaysen and Eeg-Larsen

(1953), Harrison and Harrison (1963a), Rasmussen and DeLuca (1963), DeLuca (1967) and Norman (1968).

The action of vitamin D on bone has been investigated in an attempt to see whether it directly influences bone metabolism.

The mineral content of bone has been generally regarded as divided into 2 compartments, the readily exchangeable fraction which is in rapid equilibrium with the bathing fluid, and the "non-exchangeable" fraction which, according to Rasmussen and DeLuca (1963), is in a slow metabolically-regulated equilibrium with the bathing fluid.

Vitamin D has been shown to influence the exchange in bone in which $^{45}\text{Ca}^{2+}$ had been incorporated 2 weeks before administration of vitamin D. The administration of vitamin D to vitamin D-deficient rats on low Ca^{2+} diet lead to mobilization of labelled Ca^{2+} from bone, and an elevation of serum Ca^{2+} , which could be explained by a stimulation of the resorption of bone (Carlsson, 1952). There may be an effect of vitamin D on bone resorption, through a secondary effect, via that of parathyroid hormone (PTH), since there is a lack of Ca^{2+} mobilizing action in vitamin D-deficient rats after administration of parathyroid extract (PTE) (Bernstein, Kleeman, Dowling and Maxwell, 1962). Harrison and Harrison (1960) suggested that the action of PTH upon bone resorption depends on an adequate supply of vitamin D. Thus from data available it seems that there is a synergistic effect between the two agents on the resorption process. This mobilization of bone mineral plays a significant role in supplying Ca^{2+} and phosphate to the blood serum.

The rate and amount of deposition of $^{45}\text{Ca}^{2+}$ in rachitic and

normal chick tibia after an intramuscular (Migicovsky and Emslie, 1950; Migicovsky and Jamieson, 1955) or an intravenous (Coates, Harrison and Holdsworth, 1961) injection of $^{45}\text{Ca}^{2+}$ were found to be the same. There was some indirect evidence that vitamin D has no effect upon retention of Ca^{2+} in femurs and incisors of rats fed a low or high Ca^{2+} diet after orally administered $^{45}\text{Ca}^{2+}$ (Harrison and Harrison, 1950). An effect of vitamin D has been noted on the mobilization of skeletal $^{45}\text{Ca}^{2+}$ (Thornton, 1970). The latter response appeared to be independent of PTH, was functional in hypocalcaemic chicks, and occurred in growing bone as well as in tissue in which growth has been arrested. These results suggest that vitamin D has a direct effect on bone.

Cells of rat cartilaginous epiphysis have been shown to rapidly incorporate isotopic Ca^{2+} . Mitochondria from these cells have shown a gradient of electron dense granules. These granules were shown to be in low number in the zone of proliferation and increase in number in successive zones, and were low again in the zone of provisional calcification. This suggests that intracellular Ca^{2+} leaves the cell at this site (Martin and Matthews, 1969; Matthews, Martin and Collins, 1970). This mitochondrial granule gradient was absent in the rachitic rat growth plate and was re-established following supplementation of the diet with vitamin D and phosphate. Osteoblasts in osteoid did not possess a higher number of mitochondrial granules than adjacent flat osteoblasts (mature mineralized bone). Osteoblasts were shown to possess an intracellular mitochondrial granule gradient. The mitochondria adjacent to resorbing surfaces

were filled with granules, while those at vascular surfaces were free of granules (Matthews, Martin and Collins, 1970).

Because of the difficulty of differentiating between effects of vitamin D and PTH, in vitro studies were attempted. Studies on rat calvaria in vitro by Au and Barter (1966), have shown that giving vitamin D in vivo before removing the bone, has a direct effect on bone metabolism, by possibly altering the bone-blood barrier so that Ca^{2+} can diffuse into the bone cells. Vitamin D caused more $^{45}\text{Ca}^{2+}$ to be released to the medium, while less lactate was released. There was no significant difference in the medium PO_4^{3-} concentration. The effect of vitamin D was apparent in calvaria from PTX rats and in calvaria from rats with low serum Ca^{2+} produced by low Ca^{2+} diet.

Paterson and Fourman (1968) found that vitamin D caused an increase in incorporation of ^{14}C proline into collagen hydroxyproline of rachitic chick bone in vitro and in vivo. This indicates an increased rate of collagen synthesis and cellular activity in rickets. In contrast Canas, Brand, Neuman and Terepha (1969) reported that cortical bone from rachitic chicks showed a decreased level of ash and organic material. Treatment of the rachitic chicks with 8 I.U./day vitamin D_3 restored normal composition within 7 days. Specific activity of bone hydroxyproline, after intraperitoneal injection of $[^3\text{H}]$ proline, showed 2 fold increase over rachitic control during the first 4 days of vitamin D treatment. Hydroxyproline specific activity was normal at 8 days. The increased incorporation of $[^3\text{H}]$ proline into bone collagen was observed as early as 12 hours after vitamin D and before a rise in serum Ca^{2+} towards normal levels. The results showed that

there was an early effect of vitamin D on rachitic chicks in stimulation of bone collagen synthesis, which provides new matrix for the deposition of minerals made available by the renewed absorption of gut Ca^{2+} .

Baylink, Stauffer, Wergedal and Rich (1970) report that in vitamin D-deficient rats, the total rate of formation of osteoblastic matrix was 20% less, and the total osteoclastic bone resorption rate was 80% more than in pair fed normal control rats. In vitamin D-deficient rats the rate of maturation of osteoid and the rate of initial mineralization were both reduced to approximately half that of normal rats. Matrix formation and mineralization were significantly correlated with the concentration of Ca^{2+} but not to the concentration of phosphate in serum.

Tissue cultures of tibia from 14 day old embryos of chicks were incubated in control medium and a medium containing vitamin D-treated serum. Prasad and Udupa (1970) found that the evidence from biochemical analysis of hexosamine, hydroxyproline, histological, histochemical, examination of length, wet and dry weights of chick embryo tibia that vitamin D produced resorption of bone directly and increased osteogenesis indirectly.

There was significant reduction in the growth of vitamin D-deficient rats as compared to replete rats, the reduction being most marked in the female rats given a diet adequate in Ca^{2+} and PO_4^{3-} for 8 weeks (Rasmussen, 1970). Serum Ca^{2+} was reduced but PO_4^{3-} was unchanged. However, the $[\text{Ca}^{2+}][\text{HPO}_4^{2-}]$ product was considered to be high enough to permit normal calcification. The degree of mineralization

of the cortical bone tissue increased according to the distance from the epiphyseal plate. However, no significant difference between vitamin D-treated and vitamin D-depleted animals was detected. It was concluded that vitamin D deficiency in rats does not prevent the formation of bone matrix, which can attain a normal degree of mineralization (Rasmussen, 1970). Thus, there is conflicting evidence for a direct effect of vitamin D on bone matrix formation.

Nagode, Haussler, Boyce, Pechet and Rasmussen (1970) were able to show that vitamin D increased the activities of alkaline phosphatase, Ca^{2+} -ATPase and pyrophosphatase in tibial epiphyseal plates after 70 hours feeding rachitic chicks with 50 I.U. of vitamin D_3 . From inhibitor studies it was deduced by these workers that these 3 enzyme activities were properties of a single molecule. Woltgens, Bonting and Bijvoet (1970a and b) also reported the same properties of pyrophosphatase and alkaline phosphatase in molars of 3 day old hamsters. They also concluded that the two activities were due to the same enzyme.

(3) The effect of vitamin D upon intestinal phosphate absorption

There has been no conclusive evidence obtained which demonstrates an unequivocal effect of vitamin D on transport of phosphate from lumen of the small intestine. This topic has been reviewed by Nicolaysen and Eeg-Larsen (1953), Harrison and Harrison (1963a), DeLuca (1967) and Norman (1968).

Dols, Jansen, Sizoo and De Vries (1937) noted no effect of vitamin D on $^{32}\text{PO}_4^{3-}$ absorption from an oral administration. Neville and Holdsworth (1968) noted that vitamin D given to rachitic chicks had no

effect upon phosphate transport from ileal loops when Ca^{2+} was absent. When Ca^{2+} was given together with PO_4^{3-} then an effect of vitamin D could be demonstrated. Hurwitz, Stacey and Bronner (1964) demonstrated that vitamin D improved Ca^{2+} absorption more than PO_4^{3-} absorption, using rachitic rats on a diet with adequate mineral content, but with rats on a rachitogenic diet where PO_4^{3-} was limited, PO_4^{3-} absorption was much greater than Ca^{2+} absorption. However, this effect of vitamin D_3 on PO_4^{3-} absorption was not as a direct consequence, as the rachitogenic diet was found to stimulate increased PO_4^{3-} absorption.

Harrison and Harrison (1961) carried out studies with everted intestinal sacs and found that the active transport of phosphate was totally dependent upon the presence of Ca^{2+} and K^+ , and independent of vitamin D status of the rat. However Kowarski and Schachter (1969) showed that vitamin D given to rats increased phosphate transport selectively in vitro in the direction, mucosa to serosa. This phosphate transport was shown to be via restricted channels in the mucosa, as less than one third of $^{32}\text{PO}_4^{3-}$ labelled phosphate was miscible with the mucosal Pi -pool. They concluded that vitamin D affected the phosphate and Ca^{2+} transport mechanism separately.

(4) Renal absorption of calcium and phosphate

This subject has been reviewed by Rasmussen and DeLuca (1963), Harrison and Harrison (1963a), DeLuca (1967) and Norman (1968).

Of the Ca^{2+} filtered by the kidney, 99% is reabsorbed in the absence of vitamin D or PTH. According to Gran (1960), vitamin D does influence the reabsorption of the remaining 1% of Ca^{2+} . This was

claimed on the basis of parenteral administration of Ca^{2+} to a rachitic and vitamin D-treated puppy.

In normal dogs, the infusion of PO_4^{3-} into the renal artery increases the mean clearance of ultrafilterable Ca^{2+} , Na^+ , K^+ and PO_4^{3-} while creatinine clearance and urine volume were unchanged. There was no significant change in the filtered load by Ca^{2+} . The mechanism of increase in Ca^{2+} excretion which PO_4^{3-} induces was probably a reduction in tubular reabsorption of Ca^{2+} (Hulley, Goldsmith and Ingbar, 1969). The peripheral vein infusion of PO_4^{3-} caused a delayed decrease in mean Ca^{2+} clearance. Therefore it was suggested that an increase in plasma PO_4^{3-} concentration caused a decrease in Ca^{2+} clearance and did so by a primarily extra-renal action.

Borle (1969c) reported that 50 ng/ml of vitamin D, in the culture medium enhanced Ca^{2+} efflux from the kidney cells but did not affect uptake.

There is considerable controversy concerning the nature of the action of vitamin D on kidney. When the effect of PTH and CT on the metabolism of vitamin D in the kidney has been determined, then the effect of vitamin D on the Ca^{2+} and PO_4^{3-} transport system in the kidney may be clarified.

(5) Action of vitamin D on the concentration of calcium and phosphate in the serum

Ca^{2+} and PO_4^{3-} are added to the serum by mobilization of these ions from bone, by absorption from the intestine, and by Ca^{2+} and

PO_4^{3-} reabsorption in the kidney. In the normal situation the product of $[\text{Ca}^{2+}] [\text{HPO}_4^{2-}]$ in the blood is maintained at a constant value.

This blood then may be saturated in respect to bone minerals. A fall in serum Ca^{2+} and PO_4^{3-} may be compensated for by a resorption of bone which released Ca^{2+} and PO_4^{3-} to serum. The resorption of bone being dependent on the vitamin D status of the animal. This was reviewed by Rasmussen and DeLuca (1963).

Chicks fed a rachitogenic diet (1.43% Ca^{2+} , 1.10% PO_4^{3-}) exhibit a low serum Ca^{2+} and a low bone ash, but have significantly higher serum phosphate than chicks on a normal diet, or those that had been treated with vitamin D (Chen and Bosmann, 1964).

Hypervitaminosis raised serum Ca^{2+} levels, while vitamin D deficiency lowered serum Ca^{2+} concentration and raised serum PO_4^{3-} of rats (Haskim and Clark, 1969). The occurrence of hypocalcemia in vitamin D-deficient rats was interpreted as a consequence of both reduced Ca^{2+} absorption and of inadequate resorptive response of bone cells to homeostatic stimuli, such that although bone resorption was greater than normal, it did not adequately compensate for the reduced intestinal absorption (Baylink et al, 1970).

Hypocalcemia was evident at 14 days in chicks on vitamin D_3 -deficient diets (0.8 or 1.0% Ca^{2+}). This was corrected by vitamin D dosage within 48 hours in chicks on 1% Ca^{2+} diet. The vitamin D effect on Ca^{2+} mobilization from bone of chicks on the lower Ca^{2+} diet (0.8%) persisted throughout the period of study, with only partial correction of hypocalcaemia. Thornton (1970) concluded that vitamin D first acted upon the bone Ca^{2+} mobilization and then the gut Ca^{2+} absorption, to correct the

hypocalcemia.

In rats on a phosphate-depleted diet, the serum PO_4^{3-} level fell to less than 1.0 mg/100 ml and diffusable serum Ca^{2+} either remained unchanged or rose transiently (Coburn and Massry, 1970). This would mean that the $[\text{Ca}^{2+}] / [\text{HPO}_4^{2-}]$ would not be constant but would be well below the normal level.

An involvement of CAMP in the hypercalcemic effect of vitamin D was suggested by Avery and Bell (1970) on evidence that imidazole, an activator of phosphodiesterase diminishes the rise of serum Ca^{2+} caused by vitamin D. The inhibitory effect of imidazole was observed in either intact or thyroparathyroidectomised (TPTX) rats. The hypocalcemic effect of imidazole was diminished by simultaneous injections of DB CAMP or theophylline.

(6) Action of vitamin D on citrate metabolism

There are several reviews on this subject by Nicolaysen and Eeg-Larsen (1953), Harrison and Harrison (1963a), DeLuca (1967), Norman (1968) and DeLuca (1971).

The effect of vitamin D on citrate metabolism is to increase (a) citrate content of bone (Norman and DeLuca, 1964; Harrison and Harrison, 1963a), (b) the urinary excretion of citrate (Bellin and Steenbock, 1952), (c) the serum citrate content (Harrison and Harrison, 1963a), (d) the citrate content of the kidney, heart and small intestine (Steenbock and Bellin, 1953; DeLuca, Gran and Steenbock, 1957; DeLuca, Gran, Steenbock and Reiser, 1957; DeLuca and Steenbock, 1957; DeLuca, 1965) and is (e) to decrease the uptake of citrate into the mitochondria of the kidney. It was

suggested that citrate appeared to cure rickets in rats fed on cereal diets due to the complexing of intestinal Ca^{2+} with citrate, thus preventing the formation of Ca^{2+} phytate (Pileggi et al, 1956).

The suggestion was made that vitamin D induces an increase in citrogenase activity, particularly in bone cartilage, (Meyer, Bolen and Antin, 1959) and removes the block in glycogen metabolism. These workers were unable to observe any increase in lactic, pyruvic or acetic acid production in vitamin D-deficient animals.

Prior administration of vitamin D to rachitic rats has been shown to reduce citrate and isocitrate oxidations in kidney homogenate (DeLuca, Gran and Steenbock, 1957) and isolated kidney mitochondria (DeLuca and Steenbock, 1957), but not by liver homogenate or mitochondria (DeLuca, Gran, Steenbock and Reiser, 1957). This diminished oxidation of citrate by the vitamin D administration was explained by the inhibition of citrate penetration into the mitochondria, due to vitamin D protecting the structural integrity of these organelles (DeLuca, Reiser, Steenbock and Kaesberg, 1960).

The local concentrations of citric acid induced by vitamin D and also by PTH may be responsible for mobilization of bone as postulated by Carlsson and Hollunger (1954) and Neuman and Neuman (1958). The increase in extracellular citrate content by vitamin D was shown to be prevented by panthothenic acid, or pyridoxine deficiency, or by cortisol treatment without interfering with the action of the vitamin on the serum Ca^{2+} (Harrison, Harrison and Park, 1957; 1958; Guroff, DeLuca and Steenbock, 1963). It therefore appears that the action of vitamin D on citrate metabolism may not be a primary action,

but may instead be a consequence of the action of the vitamin on membrane permeability of tissues generally. However, Norman and DeLuca (1964) and Kunin and Krane (1965) showed that in slices of epiphysis, cartilage, spongiosa or bone shaft, utilizing [^{14}C] acetate, that vitamin D administered to rachitic rats led to (a) an increase in the amount of radioactive citrate, (b) a decrease in the amount of radioactivity in the other organic acids of tricarboxylic acid cycle and (c) a decrease in the amount of radioactive carbon dioxide produced. Thus vitamin D probably directly decreases the rate of conversion of citrate into subsequent intermediates in the TCA cycle.

(7) Action of vitamin D on lipid metabolism

This topic has been reviewed by Mellander (1963), DeLuca (1967) and Norman (1968).

The vitamin has been shown to stimulate the labelling of phospholipid by $^{32}\text{PO}_4^{3-}$ in the isolated intestinal mucosal cells and kidney slices, but not in liver slices. This incorporation was found to be independent of Ca^{2+} in the medium (Thompson and DeLuca, 1964). This effect was prevented by prior administration of actinomycin D (Zull, Czarnowska-Misztal and DeLuca, 1966). Hosoya, Watanabe and Fujimori (1964) and Hosoya, Moriuchi and Ooizumi (1970) obtained results in contrast to Thompson and DeLuca (1964), that indicated that Ca^{2+} alone could stimulate the incorporation of [^{14}C] serine into the phospholipid fraction during in vitro incubation of liver mitochondria obtained from vitamin D-deficient rats, and that vitamin D added in vitro would further stimulate this incorporation. The incorporation only occurred at 37°C and not at 4°C , was maximal during the first 30 minutes at

concentration of $10\text{mM}-\text{Ca}^{2+}$, was not inhibited by cyanide 2,4 DNP, arsenate, oligomycin, but was inhibited by N.E.M. (Hosoya, et al 1970). Neville and Holdsworth (1968) found no stimulation by vitamin D of $[^{14}\text{C}]$ ethanolamine and $[^{14}\text{C}]$ serine incorporation into the mucosal phospholipids of the chick ileum in vivo, and reported that Ca^{2+} was obligatory for the stimulation by the vitamin of $^{32}\text{PO}_4^{3-}$ incorporation into intestinal phospholipids. Hubscher (1962) in subcellular studies not concerned with vitamin D, found that the incorporation of $[^{14}\text{C}]$ serine into lipid fraction was greatly stimulated by the presence of Ca^{2+} .

Thus the mechanism of action of vitamin D on phospholipid metabolism is likely to be an indirect effect, due to its action on Ca^{2+} and PO_4^{3-} metabolism.

THE ROLE OF PARATHYROID HORMONE AND CALCITONIN ON CALCIUM HOMEOSTASIS

In the past decade, a tremendous upsurge of interest in Ca^{2+} homeostasis has occurred and a number of excellent reviews have been published outlining the development in this period (Hirsch and Munson, 1969; Potts and Deftos, 1969; Rasmussen and Pechet, 1970; Copp, 1970; Potts, 1970; Talmage, Cooper and Park, 1970; Kleeman, Massry and Coburn, 1971). This may be attributed perhaps to the remarkable progress made in the chemistry and physiology of calcitonin and parathyroid hormone and the recognition of adenylyl cyclase, an active intermediate in the PTH response. Further, the isolation and characterization of vitamin D metabolites has given the opportunity for a greater appreciation of the relationship existing between these hormones in the control of homeostasis.

(1) Physiological response to Parathyroid Hormone

Although the mechanism of PTH action is far from lucid, it is evident that a multitude of metabolic activities can be associated with the influence of this hormone.

It is generally accepted that the principal function of PTH is to promote the maintenance of a normal blood Ca^{2+} level, to assure the availability of this ion at concentration optimal for the many metabolic activities it influences, including skeletal remodelling. The basis for this hormone function is its action upon (1) bone; causing the release of Ca^{2+} into the extracellular fluid phase (Talmage, 1967; Belanger and Rasmussen, 1968), (2) kidney; promoting the very rapid urinary excretion of PO_4^{3-} and perhaps also restricting the urinary excretion of calcium (Munson, 1955; Biddulph, Hirsch, Cooper and Munson, 1970), and (3) the intestine; to enhance intestinal Ca^{2+} transport (Dowdle, et al, 1960).

As far as cellular activities are concerned, PTH is known to (1) increase the number and activity of osteoclasts (Goldhaber, 1965; Talmage, Doty, Cooper, Yates, and Neunschwander, 1965; Belanger 1965), (2) increase RNA synthesis in osteoclasts (Talmage et al, 1965; Owen and Bingham, 1968), (3) decrease the synthesis of osteoblasts (Owen and Bingham, 1968), (4) increase fluxes of Ca^{2+} and PO_4^{3-} ions in and out of cultures of HeLa kidney cells (Borle, 1968; 1969a and b) and bone cells (Raisz, 1970a and b), (5) increase adenyl cyclase activity in the kidney and bone cultures (Chase and Aurbach, 1967; 1968a and b; Melson, Chase and Aurbach, 1970) and urinary excretion of CAMP from the kidney (Chase and Aurbach, 1967 ; 1968a and b), (6)

increase glucolysis in bone cultures (Borle, Nichols and Nichols, 1960), (7) increase lipid content of plasma (Notario and Larriza, 1956), (8) suppress collagen synthesis in bone (Gaillard, 1961) and increase hydroxyproline excretion by the kidney (Flanagan and Nichols, 1965 a and b), (9) increase the mitotic rate in the HeLa cell culture and DNA synthesis in bone culture (Borle and Neuman, 1965), (10) effect the activities of the transaminases, succinic dehydrogenase, isocitric dehydrogenase aconitase, collagenase, pyrophosphatase, proteolytic and lysosomal enzymes and organic acid production (see review by Talmage, Cooper and Park, 1970). The resultant effect of these altered responses causes a net shift in equilibrium towards an increase in bone resorption with transfer of Ca^{2+} and phosphate into extracellular fluid. The controlling influence on PTH secretion is the concentration of Ca^{2+} in plasma. More PTH is secreted when the concentration falls below the normal level.

(2) Mechanism of action of Parathyroid Hormone

The mechanism whereby PTH exerts its effect on the end organs has received considerable attention. Evidence indicates that the adenyl cyclase-3'5' adenosine monophosphate system may be the mediator of the physiological action of PTH on bone, kidney and the intestine (Rasmussen, Pechet and Fast, 1968; Raisz, 1970b; Harrison and Harrison, 1970; Wells and Lloyd, 1970; Aurbach and Chase, 1970). Current theory suggests that the primary action of PTH is to increase the intracellular Ca^{2+} content. The regulation of the distribution of this intracellular Ca^{2+} between connecting intracellular compartments may be controlled by CAMP (Borle, 1969d). Thus the hormone may be effective in promoting

the movement of Ca^{2+} from the extracellular fluid phase by increasing the permeability of cell membrane to this ion. As a consequence it stimulates the increased CAMP formation and affects other enzyme activities that are influenced by an alteration in Ca^{2+} concentration. The stimulation of adenylyl cyclase activity by PTH increases the level of CAMP in the kidney and urine (Chase and Aurbach, 1967; 1968a). Supporting evidence has been derived with CAMP and dibutyryl derivatives of CAMP to show that they exhibit a similar action to that of PTH (Rasmussen, Nagata, Feinblatt and Fast, 1968; Rasmussen, Pechet and Fast, 1968; Vaes, 1968; Raisz and Klein, 1969).

(3) Parathyroid Hormone effect on other ions

It has been shown PTH also increases the excretion of Na^+ , K^+ , HCO_3^- ions while it decreases excretion of H^+ , NH_4^+ (Arnaud, Tenenhouse and Rasmussen, 1967) and Mg^{2+} (MacIntyre, Boss and Troughton, 1963; Massry, Coburn and Kleeman, 1969). It was demonstrated that there is an inverse relationship with plasma Mg^{2+} level and parathyroid activity and PTH secretion (Gitelman, Kukolj and Welt, 1968; Buckle, Care, Cooper and Gitelman, 1968; Sherwood, 1970; Massry, Coburn, Kleeman, 1970; Sherwood, Herman and Basset, 1970). However, the exact role of PTH and regulation of these ions in the plasma and kidney are not clear.

(4) Physiological response to calcitonin

Since the recognition of this hormone in 1961, calcitonin has been isolated, its structure determined and synthesised. Its distribution, storage and physiologic mode of action has also been established. It appears to be mainly located and stored in large

amounts in the parafollicular or "C" cells of the mammalian thyroid and in the ultimobranchial glands of many species (Copp 1970).

The most striking response of calcitonin is the lowering of plasma Ca^{2+} concentration and this has been best demonstrated in young and growing animals, particularly when bone remodelling is in progress (Copp, 1970). The response was independent of the parathyroid glands, intestinal tract, liver, kidney and the Ca^{2+} and PO_4^{3-} concentration in soft tissue (Hirsch and Munson, 1969; Potts, 1970; Copp, 1970). Calcitonin appeared to exert its influences primarily by inhibiting bone resorption (Hirsch and Munson, 1969; Copp, 1970; Potts, 1970).

Calcitonin has been shown to (1) inhibit activity of the osteoclasts (Foster, Doyle, Bordier and Matrajt, 1966) (2) decrease the number of osteoclasts (Foster et al, 1966) and (3) decrease activity of osteocytes (Belanger and Rasmussen, 1968). Thus calcitonin reduced the Ca^{2+} mobilizing effect of PTH (Hirsch and Munson, 1969; Potts, 1970; Copp, 1970; Kleeman, Massry and Coburn, 1971). However Frankel and Yasumura (1971) have demonstrated, with high dose of vitamin D_3 given to rats, that endogenous calcitonin does not fully eliminate the increase in the rate of bone resorption.

(5) Mechanism of action of calcitonin.

The biochemical mechanism affecting bone resorption is unknown. Morii and DeLuca (1967) found calcitonin does not require vitamin D to act and there is strong evidence against its having a direct effect on either protein synthesis or the adenyl cyclase system (Chase, Fedak and Aurbach, 1969; Kleeman, Massry and Coburn, 1971).

Borle (1969d) and Raisz (1970b) have shown that calcitonin inhibits active efflux of $^{45}\text{Ca}^{2+}$ from kidney cells and from bone cells in tissue culture.

Calcitonin has been detected in the plasma of many species when the plasma Ca^{2+} levels are normal (Foster, 1968). This indicates that calcitonin must be continuously secreted both in the absence of a hypercalcemic stimulus and during normal and mild hypocalcemic states (Klein and Talmage, 1968). The rate of secretion of calcitonin has been shown to be directly proportional to blood Ca^{2+} concentration.

(6) The relationship of calcitonin, parathyroid hormone and vitamin D

Even though a great deal of work has been done on the effect of these three agents on target organs, very little conclusive evidence has been obtained.

The topic has been comprehensively reviewed by Potts and Deftos (1969). They maintain that the continued secretion of calcitonin and parathyroid hormone at normal blood Ca^{2+} concentration, the rapid increase in calcitonin secretion with hypercalcemia and the rapid increase in PTH secretion with hypocalcemia, all assure extremely precise modulation of blood calcium concentration through the regulation of bone resorption. This controls the supply of Ca^{2+} liberated from bone into the extracellular fluid (Copp, 1970; Potts, 1970). The PTH-CT control of Ca^{2+} homeostasis, by a fine negative feed back system, prevents any overshoot under normal conditions.

Vitamin D can replace PTH in the maintenance of a normal plasma Ca^{2+} concentration under steady state conditions, but when vitamin D-treated animals are maintained on a low Ca^{2+} diet,

parathyroidectomy produces a drop in the plasma Ca^{2+} and a delayed rise in plasma phosphate which results in death. However, an injection of PTH prevented both of these changes (Harrison and Harrison, 1970), while the administration of PTH to vitamin D-deficient rats had little effect on plasma Ca^{2+} level (Harrison, Harrison and Park, 1958; Rasmussen, DeLuca, Amaud, Hawker and Von Stedling, 1963; DeLuca and Sallis, 1965). Thus PTH and vitamin D act synergistically to increase Ca^{2+} and phosphate in the plasma.

The action of calcitonin in lowering the serum Ca^{2+} has been demonstrated to be independent of vitamin D (Gudmundson, MacIntyre and Soliman, 1966; Morii and DeLuca, 1967) and phosphate levels of plasma (Morii and DeLuca, 1967). However the action of calcitonin in TPTX animals is prolonged while the injection of the two hormones at the same time reduces the effectiveness of calcitonin (Morii and DeLuca, 1967). In the absence of both CT and PTH, a pharmacological dose of vitamin D was able to maintain serum Ca^{2+} at a normal level. Endogenous calcitonin did not fully eliminate the hypercalcemia produced by pharmacological doses of vitamin D (Frankel and Yasumura, 1971).

A large dose of PTH stimulated excretion of PO_4^{3-} from the kidney in the absence of vitamin D, but with the administration of vitamin D, the effective dose of the hormone required to produce a similar response on renal tubular phosphate excretion was reduced (Rasmussen and DeLuca, 1963). Borle (1969d) has shown vitamin D and calcitonin to act synergistically in the inhibition of Ca^{2+} efflux from kidney cells in culture. An increase in urinary hydroxyproline occurs with vitamin D-deficient rats after

TPTX in contrast to vitamin D-replete rats (Rasmussen and Feinblatt, 1971). This hydroxyprolinuria was increased further by PTH or CT infusion. However, PTH increased hydroxyprolinuria in vitamin D-fed animals while CT caused a significant decrease. In the vitamin D-deficient animals, both PTH and CT caused a decrease in urinary Ca^{2+} excretion and increased the positive Ca^{2+} balance. In vitamin D-fed animals, CT had similar effects, but PTH caused a significant increase in Ca^{2+} excretion and negative Ca^{2+} balance. In young animals injected with $^{45}\text{Ca}^{2+}$, maintained on a vitamin D-deficient diet for 3 weeks, then TPTX, the excretion of Ca^{2+} was relatively constant and the specific activity of urinary Ca^{2+} declined slightly. When PTH was given, both total and radioactive Ca^{2+} excretion diminished for the first 3 hours and then gradually rose over the next 4 hours to become greater than normal. When CT and PTH were both given, the total Ca^{2+} excretion fell and remained below the control rate. The specific activity rose markedly during the first 2 hours and then rapidly fell to values below the control level.

PTH and 25 hydroxycholecalciferol (25 HCC) (an active metabolite of vitamin D_3) appeared to act synergistically on bone. 25 HCC stimulated bone resorption in tissue culture in a manner very similar to that produced by PTH (Trummel, Raisz, Blunt and DeLuca, 1969). PTH has been shown to stimulate the adenocyclase system in bone while vitamin D has no effect on the system. Raisz (1970b) postulated that these two agents work at separate but linked sites, in that PTH controls Ca^{2+} entry into the cell and vitamin D_3 , or its active metabolite, controls the entry of Ca^{2+} into the nucleus, perhaps controlling transcription

and cellular transformation. Vitamin D can replace PTH in producing a normal skeletal response to hypocalcemic stress. However, Rasmussen and Feinblatt (1971) have proposed that vitamin D acts by controlling a step or steps in PTH induced bone resorption after adenylyl cyclase activation. A large dose of vitamin D, however, seems to mimic the action of PTH plus vitamin D upon bone resorption (Rasmussen and DeLuca, 1963).

Other experimenters have demonstrated that when PTH was administered to vitamin D-deficient rats there was little effect on intestinal Ca^{2+} transport (Harrison, et al, 1958; Dowdle et al, 1960; Toverud, 1964). Moreover vitamin D-deficient animals have a reduced absorption of Ca^{2+} despite hypersecretion of PTH (Harrison and Harrison, 1970). They have also shown that CAMP in vitamin D-treated rats enhanced intestinal Ca^{2+} transport. Hypoparathyroid animals receiving a normal intake of vitamin D have defective intestinal Ca^{2+} transport and a large dose of vitamin D could be shown to correct the abnormality according to Harrison and Harrison (1970). Thus, vitamin D would appear to be required for the mediation of PTH effect on the intestinal tract. Calcitonin on the other hand has been shown to have no direct effect on intestinal Ca^{2+} transport (Krawitt, 1967a and b; Winter, Morova and Simon, 1970).

In conclusion, it can be said that vitamin D acted synergistically with PTH to promote the resorption of bone, thus causing the plasma Ca^{2+} and PO_4^{3-} concentration to rise. Both these agents act synergistically on the intestine and kidney. They act at the intestine and enhance Ca^{2+} transport, thus raising the plasma Ca^{2+} .

level. Secondly they act on the kidney to increase excretion of PO_4^{3-} . The influence of calcitonin on plasma Ca^{2+} and PO_4^{3-} level and bone resorption appeared to be independent of vitamin D, but it does appear that they both act upon the Ca^{2+} efflux from kidney cells. PTH and CT secretion are controlled by the plasma Ca^{2+} concentration which is in turn dependent upon the vitamin D status of the animal.

These three agents are thus interwoven into a finely controlled negative feed-back system. However, the formation of CAMP as an intermediate in the response to PTH stimulation of the kidney and bone appears to be independent of vitamin D and calcitonin regulation. This interwoven control on plasma Ca^{2+} and PO_4^{3-} level may be enlightened further with experiments on the control of the metabolism of vitamin D into its metabolites, especially in the kidney. At the moment there is interest in the effect of Ca^{2+} plasma levels upon the formation of metabolites 1,25 dihydroxycholecalciferol (1,25 DHCC) and 24,25 dihydroxycholecalciferol (24,25 DHCC). One of these metabolites may control bone resorption and Ca^{2+} intestinal absorption, and the other controls kidney excretion of Ca^{2+} and PO_4^{3-} .

VITAMIN D METABOLISM

The topic of vitamin D metabolism has been extensively reviewed by Rasmussen and DeLuca (1963), DeLuca (1967), Norman (1968), Wasserman (1968), DeLuca (1969b), Wasserman and Corradino (1971) and DeLuca, Elunt and Rikkers (1971).

During the 1920's and 1930's the main facts about the formula, and its physiological effects on Ca^{2+} and Pi metabolism were elucidated (reviewed by DeLuca, 1967; Potts and Deftos, 1969). Since that time,

there has been steady, but no intense interest in the vitamin until the last six years, when exciting new discoveries have been made.

The main problem in describing these compounds in relation to the vertebrate kingdom is whether to call them "vitamins" or "hormones", as they are metabolized within the body and transported via the blood stream to target organs. Thus they are intermediate between a vitamin and a hormone, for they are metabolized in the body, and converted to a more active hormonal form before direct transportation to the target organ.

It has been known for a long time that sterols can be synthesised by plant and animal tissues. The synthesis of 7 dehydrocholesterol in animals and ergosterol in plants has been shown to initiate from acetyl CoA (reviewed by DeLuca, Blunt and Rikkers, 1971). On irradiation by U.V. light the provitamin was converted to vitamin D. To a lesser extent *ergocaliferol* was formed from irradiation of ergosterol (previtamin D₂).

Vitamin D has two important effects: the curing of rickets, and bone mobilization. However dihydrotachysterol possesses only the bone mobilizing effect of vitamin D. In this regard its potency is equal to that of vitamin D₃ and vitamin D₂. The structure of ergocalciferol (vitamin D₂) differs from that of cholecalciferol only by a double bond at 22,23 position and an extra methyl group at position 24. The antirachitic activity of vitamin D₃ and vitamin D₂ is equal in rats but not in chickens. Vitamin D₂ possesses one tenth the activity of vitamin D₃ in curing rickets in chickens (Chen and Bosmann, 1964). It has been established that one I.U. of vitamin D₂

or D_3 is equal to 0.025 μg in the rat and a physiological dose is considered to be 1-200 I.U. There have been biological methods developed to detect as little as 0.5 - 5 I.U. of vitamin D administered to vitamin D-deficient animals (Norman, 1966a; Schachter, Kowarski and Reid, 1967).

Dihydrotachysterol series 2 and 3 have been shown to be as active as vitamin D_3 and superior to D_2 in increasing Ca^{2+} absorption by chick (Dowdle et al, 1960; Sallis and Holdsworth, 1962a; Bosmann and Chen, 1966). While in rats the antirachitic activity of DHT_2 and DHT_3 were far less potent. It is now known to possess a greater potency in bone mobilization than ergocalciferol in normal rats (Kaye, Just and Wilson, 1971).

The synthetic vitamin D_4 (22,23 Dihydroergocalciferol) possesses one I.U./0.033 μg (30,000 I.U./mg) of antirachitic activity in rat and one I.U./0.25-0.125 μg (4,000-8,000 I.U./mg) of activity in chicks (DeLuca, Weller, Blunt and Neville, 1968).

(1) Absorption, transport and excretion of vitamin D

The site of absorption of vitamin D has been established as the lower half of the small intestine in man (Avioli, 1969) and in rats (Kodicek and Ashby, 1954, 1960a; Norman and DeLuca, 1963). Schachter, Finkelstein and Kowarski (1963) noted that vitamin D entered the lymphatic system in chylomicrons within 20 minutes after an oral administration and afterwards reached the blood stream.

The phenomena of esterification of vitamin D during absorption was studied by Bell (1966) who found that of the $[^{14}\text{C}]$ vitamin D absorbed, very little was esterified in comparison to cholesterol.

This esterification of vitamin D is not obligatory for absorption or transportation. However, the absorption of vitamin D₃ and cholesterol were found to be further enhanced by the duodenal administration of mixed micellar solutions of either linoleic or palmitic acid as compared to taurocholate (Thompson, Ockner and Isselbacher, 1969). The increase in vitamin D and cholesterol absorption was reported linearly to lymph triglycerate level. Bell and Bryan (1969) observed that vitamin D₃ oleate was not absorbed as well as the free vitamin. It appears that the vitamin D ester was hydrolysed before absorption and then esterified by rat pancreatic juices. The thoracic duct of the lymph system was found to transport 43% of an oral dose of vitamin D in 12 hours. Only 4% of the transported vitamin was esterified (Fraser and Kodicek, 1966).

Once the vitamin is in the blood stream as chylomicrons, there is an association with lipoproteins of the blood (Norman and DeLuca, 1963; Schachter, Finkelstein and Kowarski, 1964; Neville and DeLuca, 1966; Rikkers and DeLuca, 1967). Rikkers and DeLuca (1967) found that 50% of the radioactive vitamin D injected into the blood stream was associated with lipoprotein fractions and then shifted progressively to alpha globulin fraction. Thus it appears that this non-lipoprotein is the prime carrier of a physiological amount of vitamin D and its metabolites. The association of vitamin D with this protein is fairly strong, it is time dependent and is saturated at very low doses of vitamin D (Chen and Lane, 1965).

There is general agreement that vitamin D and its metabolites,

when given in larger than physiological amounts, are excreted into the small intestine via the bile, finishing up in the faeces (Kodicek and Ashby, 1960b; Norman and DeLuca, 1963; Schachter et al, 1963; Avioli, Lee, MacDonald, Lund and DeLuca, 1967; Bell and Kodicek, 1969). The bile excretion is established to be as a glucuronide of the vitamin (Avioli, et al, 1967; DeLuca, 1967; Imrie, Neville Snellgrove and DeLuca, 1967; Bell and Kodicek, 1969).

(2) The site of storage and distribution of vitamin D in the body

The distribution of vitamin D has only just recently been shown at physiological levels by means of high specific activity [^3H] and [^{14}C] vitamin D's. The progress in this area of vitamin D metabolism has been particularly rapid in the last few years. This topic has been reviewed by DeLuca (1967), Norman (1968), Jowsey (1969) and Wasserman and Corradino (1971).

When large doses of vitamin D, in excess of the amount required for a physiological response, were given, the bulk of the vitamin or metabolite in the tissues and excreta was represented as storage forms and by degradation products with little or no function.

Biological assays first used to detect vitamin D were difficult and insensitive. Even with such biological methods, Morgan and Shimotori (1943) and Cruickshank and Kodicek (1953) found that vitamin D activity was present in kidney, heart, large intestine, brain, lung, spleen, muscle, fat, skin and hair. Of the 40,000 I.U. dose of vitamin D administered to the dog, only 10% was recovered in the body (Morgan and Shimotori, 1943). However Cruickshank and Kodicek (1953) and Cruickshank, Kodicek and Armitage (1954) were able to

retrieve 25% of the dose given to rats. Of this, 20% was present in the muscle after 2 days.

Kodicek (1955) prepared the radioactive vitamin D by growing yeast on radioactive acetate (^{14}C). From the yeast he extracted the ergosterol and irradiated this with U.V. light to produce ergocalciferol. After a large intravenous dose of this low specific activity [^{14}C] vitamin D₂, Kodicek (1963) reported it located in intestine, kidney, bone, muscle and liver. Sixty to eighty percent of the radioactivity, 20 minutes after an intracardial dose, and 8-10% 5 hours after an oral dose, were associated with the liver (Kodicek, Cruickshank and Ashby, 1960; Kodicek and Ashby, 1960b). After a large oral dose of vitamin D, the vitamin accumulated in liver. The amount increased with time (Norman and DeLuca, 1963). The proportion of vitamin D retained in the liver was approximately half the total retained by the body. This was irrespective of the size of the dose, 4-40,000 I.U. (Kodicek, Cruickshank and Ashby, 1960; Norman and DeLuca, 1963; Neville and DeLuca, 1966; DeLuca et al, 1968). It is generally agreed that vitamin D-deficient animals are able to assimilate more vitamin D than normal animals (Norman and DeLuca, 1963).

Using [^3H] vitamin D, Norman and DeLuca (1963), Norman (1966a), Haussler and Norman (1967), showed that the distribution of 500 I.U. in an intracardial or oral dose was a function of time. It, too, was rapidly accumulated by the liver, but after 24 hours the radioactivity was widely distributed. Of the dose administered, up to 94% of the radioactivity was recovered. Bosmann and Chen (1965) showed a wide distribution of radioactivity in chick after 1100 I.U. of [^{14}C] vitamin

D dose. The small intestine (Bosmann and Chen, 1965) and bones (Kodicek and Ashby, 1960) of rachitic animals contained more radioactive vitamin D₃ than vitamin D-treated chicks. Whereas the liver, caecum and large intestine contained less radioactive vitamin D₃. Fraser and Kodicek (1965; 1966; 1968a) reported the formation of a number of esters of vitamin D in vitro in rats 24 hours after a dose of [³H]cholecalciferol (CC). These esters were found in the intestine, liver (5-11%), and kidney (4.5-20%). The proportion of saturated fatty acid esters was higher in the kidney than the liver. Also the same percentage of vitamin D esters was found after 2 different dosage levels of the vitamin. More recently Fraser and Kodicek (1968b) have demonstrated that the total vitamin D in the liver after an oral dose decreases in an exponential fashion from 19% of the dose at 5 hours to 0.6% at 72 hours. In contrast the esters remained at a fairly constant low level. At 72 hours the esters represented 6% of the total vitamin D in the liver. In the kidney the concentration of esters increased slowly until 48 hours, then decreased to 10% of the total vitamin D at 72 hours. The small intestine contained a higher concentration of esters at 10 hours than at a later time.

Mayer and Schaefer (1969) found that 4 days after a 4,400 I.U. injected dose of [¹⁴C]vitamin D₃ into man, that the muscle appeared to store significant amounts of vitamin D and metabolites. Unfortunately the liver content of vitamin D was not estimated. The adipose tissue of rats was found to be the storage organ. Half of this vitamin D occurred in ester form (Rosenstreich, Rick and Volwiler, 1971).

Avioli, Lee, MacDonald, Lund and DeLuca (1967), Mawer and Stanbury (1968), and Mawer, Lumb and Stanbury (1969) found that after an intravenous dose, the serum [^3H] vitamin D level in human subjects followed a biphasic curve and an exponential decay. This was similar to the findings of Ponchon and DeLuca (1969b) in rats given [$1,2,^3\text{H}$] vitamin D_3 . Rikkers and DeLuca (1967) found the largest amount of unaltered vitamin D in the blood at the time when the radioactivity was leaving the liver. They also showed that vitamin D was associated with α_2 globulin in the serum. These phenomena were consistent with uptake by the liver and a subsequent release into the blood. Drescher, DeLuca and Imrie (1969), and Rikkers, Kletziens and DeLuca (1969) noted that α globulin shows a greater affinity for binding with vitamin D_3 than for vitamin D_2 or D_4 . The addition of a hydroxyl group on the side chain of vitamin D increased this affinity. Mawer et al (1969) noted that less than 5% of the radioactivity of the dose was recovered in the urine and faeces during the decay phase. The initial rapid fall in the level of radioactivity of vitamin D in serum was largely as a result of distribution of the tracer about the body.

The true distribution and metabolism of vitamin D became possible when radioactive vitamin D of sufficiently high specific activity was synthesized so that physiological doses could be given.

A number of radioactive vitamin D were made [$1,2^3\text{H}_2$] vitamin D_3 (Neville and DeLuca, 1966), [1^3H] vitamin D_3 (Norman and DeLuca (1963), [$22,23^3\text{H}_2$] vitamin D_4 (DeLuca et al, 1968) and [U^{14}C] vitamin D_2 (Imrie, Neville, Snellgrove and DeLuca, 1967). With

doses of 10 I.U. or less of [^3H] vitamin D_3 , the spleen, lung, kidney, bone and intestine all accumulated much higher concentrations of radioactivity than muscle. However after a 4 I.U. [^3H] vitamin D dose, the muscle was shown to account for a significant percentage of the dose by sheer mass (Neville and DeLuca, 1966; DeLuca et al, 1968; Imrie et al, 1967). They attempted to compute the amount of radioactivity per cell, rather than in the noncellular fraction of bone, as it was assumed that vitamin D or its metabolites would be located in the cell where it exerted its action. A correction was made for the intestine excluding the smooth muscle. It was then possible to demonstrate that bone and the intestinal mucosa acquired the highest relative incorporation of radioactivity. This is very important, as direct action of the vitamin has been shown to occur in these two organs.

Twenty-four hours after an intracardial injection of 5 I.U. of randomly labelled [^3H] vitamin D_3 to rachitic chicks, Haussler and Norman (1967) showed that the skeleton had accumulated 0.4 I.U., the intestine 0.12 I.U., the liver 0.16 I.U., and the kidneys 0.06 I.U. The rest of the radioactivity was distributed throughout the body and excreted. Ponchon and DeLuca (1969a) also reported that the liver and kidney accumulated unchanged vitamin D_3 after 10 I.U. dose. The plasma was found to contain a large amount of the radioactivity with the highest proportion in the intestine, plasma, and skeleton after intracardial 5 I.U. dose of [$1,2^3\text{H}$] vitamin D (Holman, Mawer and Smith, 1970).

Lawson, Pelc, Bell, Wilson and Kodicek (1971) reported that

after giving a 5 I.U. [^{14}C] [^3H] dose of vitamin D_3 , the tissue distribution of vitamin D_3 in rachitic rats was similar to that found in chicks given four times that dose. The only two exceptions, were the rat kidney, with a very high concentration of vitamin D_3 ; and rat blood, with an even higher proportion of the radioactivity. Radioactivity was in the intestine and bone one hour after this dose.

(3) The metabolism of vitamin D

In the development of the metabolism of vitamin D, there have been three main contributors who used different nomenclature in their original publications. These problems have now been resolved because the compounds have been identified.

The determination of the course of metabolism of vitamin D was achieved only when high specific activity vitamin D (^3H and ^{14}C) were produced and developed (Norman and DeLuca, 1963; Norman, Lund and DeLuca, 1964; Lund and DeLuca, 1966). A silica gel chromatographic system was evolved for the separation of the metabolites. This method was later improved upon by the use of sephadex LH.

From chromatography of chloroform-methanol extracts of tissue from rat given 500 I.U. [^3H] vitamin D, several metabolites of vitamin D were found. At least three radioactive compounds were detected in the kidney, liver, intestinal mucosa, serum and bone, all of which showed some biological activity. A portion of the radioactivity was found to remain in the aqueous phase and did not cure rickets (Norman, Lund and DeLuca, 1964). The validity of using tritium as a label for vitamin D was questioned by Callow, Kodicek and Thompson (1966), who noted on giving [^3H] vitamin D_2 and [$^{24,25,26,27^3\text{H}}$], [$^{7^3\text{H}}$],

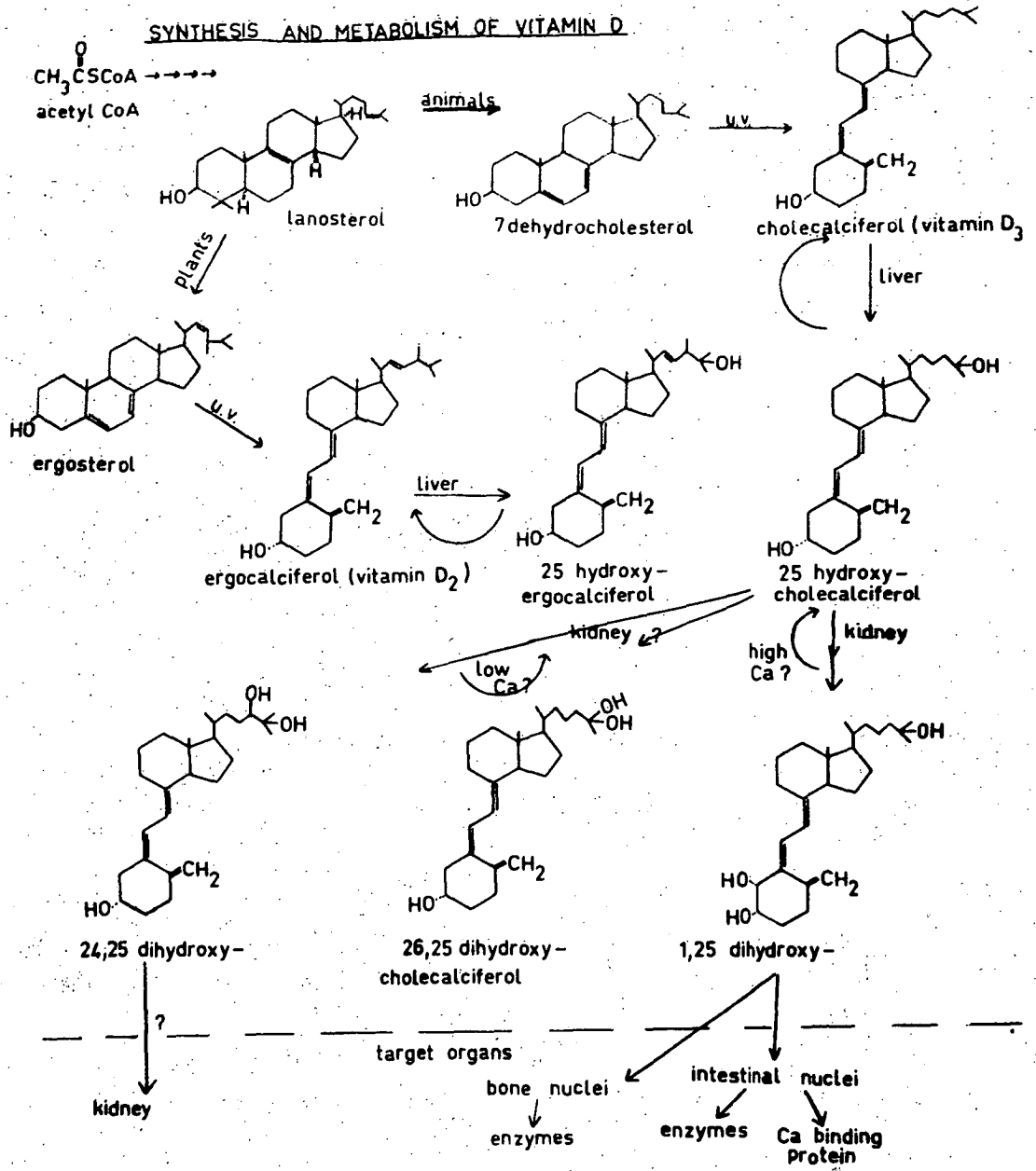
[6^3H] and [$1\alpha^3\text{H}$] vitamin D_3 to rats that there were a number of metabolites, but the vitamin had lost much of the label to the body water. It is very likely that some positions are more liable for exchange and reaction than others, and it is quite plausible in Callow's et al (1966) experiment that the position labelled was the one used to form the metabolite. By silicic acid chromatography of the tissue extracts, Norman et al (1964) and Lund and DeLuca (1966) identified the first emerging peak as esters of vitamin D (Lund, DeLuca and Horsting, 1967; Fraser and Kodicek, 1968a). While Peak II has not been identified, these peaks were never found in large amounts regardless of dose, less than 20% of 10 I.U. dose of vitamin D_3 (Lund et al, 1967). Peak III was identified as unaltered vitamin D (Lund and DeLuca, 1966; DeLuca et al, 1968). Of significance was the finding by Lund and DeLuca (1966) that when the dose level of [^3H]vitamin D was decreased from 500 to 10 I.U. the amount of one of the metabolites increased from 21 to 83% of the total chloroform soluble radioactivity. This metabolite was found to be more polar than vitamin D and possessed approximately equivalent biological activity (Norman et al, 1964; Lund and DeLuca, 1966). It was found in all tissues examined. This peak was reported to consist of at least two compounds (DeLuca et al, 1968). With smaller doses of [$1,2^3\text{H}$]vitamin D, the proportion of these metabolites found in the intestine, blood and bone increased. The rate of appearance of these metabolites rose rapidly in most tissues within four hours after administration. The isolated peak IV metabolite was readministered to vitamin D-deficient rats, and it was found to

stimulate transport in the intestine and raised the serum Ca^{2+} level (Mori, Lund, Neville and DeLuca, 1967; Ponchon and DeLuca, 1969a). This metabolite induced Ca^{2+} transport more rapidly than vitamin D. The effect was produced after 8 hours as compared to 20 hours with an equal dose of vitamin D_3 administered orally to the rat. This peak IV metabolite was subsequently isolated and purified from the plasma of pigs fed 250,000 I.U. of vitamin D_3 per day (Blunt, DeLuca and Schnoes, 1968). This compound was identified by use of gas-liquid chromatography, nuclear magnetic resonance spectrometry, cochromatography with synthesized material, mass spectrography and U.V. absorption as 25 hydroxycholecalciferol (25 HCC). This was verified by synthesis of 25 HCC (Blunt, Tanaka and DeLuca, 1968; Blunt and DeLuca, 1969). The compound corresponds to peak 4A of Haussler and Norman (1967) and Haussler, Myrtle and Norman (1968) (see fig.1). Suda, DeLuca, Schnoes and Blunt (1969a and b) have isolated and identified 25 hydroergocalciferol (25 HEC) from the plasma of pig given 500,000 I.U. of ergocalciferol/day for 26 days (see fig.1). 25 HEC was shown to exhibit antirachitic activity.

Ponchon and DeLuca (1969c) observed that $[^3\text{H}]$ vitamin D_3 -in-ethanol induced artefacts appeared during $[^3\text{H}]$ vitamin D metabolism, when compared with $[^3\text{H}]$ vitamin D_3 given in plasma. Ethanol accelerated the disappearance of the radioactivity from the plasma compartment and decreased the characteristic rebound of plasma radioactivity occurring after 1-4 hours. They suggested that ethanol interfered with (1) the diffusion to tissues due to altered permeability, (2) altered hydroxylation of vitamin D to 25 HCC, (3) decreased binding affinity

FIGURE 1

SYNTHESIS AND METABOLISM OF VITAMIN D



of plasma lipoproteins and specific carrier proteins and (4) increased cell membrane permeability.

Lawson, Wilson and Kodicek (1969a) were the first to report the loss of ^3H from positions C-1 of [^3H]vitamin D_3 in chick tissues. The simultaneous administration of two radioactive substances [$^4^{14}\text{C}$] cholecalciferol (CC) and [$^1^3\text{H}$] CC, and double labelled [$^4^{14}\text{C}$] and [$^1^3\text{H}$] 25 HCC to vitamin D-deficient chicks, showed the presence in blood, liver, intestine, kidney, bone, muscle and heart of CC, its ester, 25 HCC and a further more polar metabolite peak P. The $^3\text{H}/^{14}\text{C}$ ratio in these four radioactive components was the same as that of the dose material (4.7:1.0) with the exception of the most polar metabolite (0.4:1.0-1.8) and in all tissues examined with the exception of blood. In the chick the polar metabolite accounted for almost 70% of the radioactivity in the intestine after a dose of 0.5 μg of [$^4^{14}\text{C}$] [$^1^3\text{H}$] CC. This polar metabolite from the intestine also had the lowest $^3\text{H}/^{14}\text{C}$ ratio of all the tissues. It appeared that in chicks intestine the polar metabolite reaches a maximum concentration of 1 ng/g of tissue, a level above which it cannot be increased irrespective of the vitamin dose. After administering [$^4^{14}\text{C}$] [$^1^3\text{H}$] 25 HCC to vitamin D-deficient chicks, the metabolite with lowered $^3\text{H}:^{14}\text{C}$ ratio was detected in liver, kidney, intestine, bone, muscle and heart. None of the polar metabolites with lowered $^3\text{H}/^{14}\text{C}$ ratio was detected in blood or adipose tissue of vitamin D-deficient chicks 16 hours after dosing with either the double labelled CC or 25 HCC. When vitamin D_3 -repleted birds were dosed with radioactive CC, none of the polar metabolite with the low $^3\text{H}/^{14}\text{C}$ ratio could be detected in blood or adipose tissue. The loss of ^3H from

position C-1 was suggested to be due to hydroxylation.

Myrtle and Norman (1970) found that 15 hours after administration of 10 I.U. generally labelled [^3H] vitamin D_3 or [^{14}C] vitamin D_3 that 79% of the total radioactivity in the plasma was as peak 4A (25 HCC) and 10% as peak 4B (peak P Kodicek). Whereas in the intestine, a primary target organ for vitamin D_3 , 17% existed as 4A and 55% as 4B. These peaks 4A and 4B were also detected in rat, chick, rabbit and pig.

However, Haussler, Littledike, Boyce and Rasmussen (1970a and 1971) found that peak 4 consisted predominantly of 25 HCC as shown by counter current distribution of plasma from rachitic chicks 15 hours after an intracardial dose of 10 I.U. [$1,2^3\text{H}$] vitamin D_3 . Norman and Midgett (1970) reported on the interrelationship of the metabolites Peak 4A (25 HCC) and Peak 4B (peak P Kodicek). They stated that from the order of appearance of these metabolites in the intestine and binding of peak 4B to intestinal nuclei and chromatin, and the enhancement of Ca^{2+} transport system, that CC was first metabolized to 4A, which was then converted to 4B which in turn initiated Ca^{2+} transport response.

Peak V (Peak P Kodicek) was shown by Ponchon and DeLuca (1970) to be present in the liver for 8 hours and later became the major metabolite in the intestine within 24 hours. The presence of peak 4A and peak 4B were noted in the (a) intestinal mucosa and (b) in skeleton and (c) uterus of a laying hen after administration of generally labelled [^3H] vitamin D_3 and [^3H] dihydrotachysterol, and [$1,2^3\text{H}$] vitamin D_3 (Norman and Midgett, 1970). Unfortunately, the exceedingly low levels of 4B found in all tissues renders its chemical

characterization difficult. Massive doses of CC were not found to increase the level of most polar metabolites of vitamin D, peak P or 4B above 0.2 I.U./chick intestine (Norman, Myrtle, Nowicki and Midgett, 1971; Myrtle and Norman, 1971). During 1971, several authors isolated and/or identified this compound. Peak P, (Kodicek), peak V (DeLuca) and Peak 4B (Norman) were shown to be 1,25 dihydroxycholecalciferol (1,25 DHCC) (Holick, Schnoes and DeLuca, 1971; Holick, Schnoes, DeLuca, Suda and Cousins, 1971; Norman, Myrtle, Nowicki and Midgett, 1971; Myrtle and Norman, 1971; Haussler, Boyce, Littledike and Rasmussen, 1971; Lawson et al, 1971) (see fig. 1). Mawer, Backhouse, Lump and Stanbury (1971) were able to show the presence of 1,25 DHCC in the plasma of men after 1200 I.U. dose of $[1,2^3\text{H}] [4^{14}\text{C}]$ vitamin D_3 .

The 1,25 DHCC appeared in the intestine and bone, 1 hour after a dose of vitamin D and 30 minutes after 25 HCC administration by intracardial injection to rachitic rats (Lawson et al, 1971). There was very little 25 HCC in the intestine at any time, but in bone and blood it was a major component over the 8 hour experimental period.

(4) Biological activities of vitamin D metabolites

25 HCC was shown to possess a biological activity of 1.4-2.0 times (70-80 I.U./ μg) that of vitamin D_3 in its antirachitic activity in chickens and rats (Blunt, Tanaka and DeLuca, 1968; DeLuca, 1969a; Myrtle and Norman, 1970; Myrtle, Haussler and Norman, 1970; Kodicek, Lawson and Wilson, 1970). 25 HCC was reported to possess 1.5 times (60 I.U./ μg) the activity of vitamin D_3 or D_2 in curing rickets in rats (Suda, DeLuca, Schnoes and Blunt, 1969a and b).

Trummel, Raisz, Blunt and DeLuca (1969) reported that 25 HCC stimulates release of previously incorporated $^{45}\text{Ca}^{2+}$ from fetal rat bones in culture with doses of 0.9 - 27 I.U./ml. The effect they reported could not be produced by larger doses of vitamin D_3 . When 2.5 μg of 25 HCC was added to the arterial blood by perfusing the vitamin D-deficient intestine, it induced a rise in Ca^{2+} transport to a level similar to that of vitamin D supplemented intestine within 2 hours (Olson and DeLuca, 1969), while 1,000 I.U. of vitamin D_3 given the same way had no effect on Ca^{2+} transport level over a 4 hour period. Both these reports are inconsistent with present knowledge of the action of 25 HCC, and its conversion to 1,25 DHCC, but the results could be accounted for by the large amounts of 25 HCC used.

Early reports stated that 1,25 DHCC was only half as potent as vitamin D in stimulating intestinal Ca^{2+} absorption (Ponchon, DeLuca and Suda, 1970), half the antirachitic activity of 25 HCC, and had equal potency in bone mobilization to 25 HCC (Omdahl, Tanaka and DeLuca, 1971). Others have shown it possesses up to 13 times the activity of vitamin D and is 2.5 times as potent as 25 HCC in stimulating intestinal Ca^{2+} transport (Kodicek, Lawson and Wilson, 1970; Norman, Myrtle, Nowicki and Midgett, 1971; Myrtle and Norman, 1971; Haussler, Boyce, Littledike and Rasmussen, 1971; Omdahl, Holick, Suda, Tanaka and DeLuca, 1971b). Vitamin D and 25 HCC appear to produce maximum stimulation of Ca^{2+} transport within 24-48 hours, while with 1, 25 DHCC the stimulation reaches a maximum at 5-12 hours (Haussler et al, 1970a and 1971; Norman et al, 1971; Myrtle and

Norman, 1971; Omdahl et al, 1971b). However Kodicek et al (1970) noted that 125 ng (5 I.U.) of 1,25 DHCC had 50% greater effect in rising blood Ca^{2+} than vitamin D_3 . After 24 hours serum Ca^{2+} concentration was unaffected by 125 ng of 1,25 DHCC while larger dose (1500 ng) of CC increased serum Ca^{2+} concentration. The concentration of 1,25 DHCC in the intestine was found to be constant at 13 pmoles per chick, regardless of vitamin dosage. A sensitive in situ technique revealed that 1,25 DHCC (325 pmoles) stimulated intestinal Ca^{2+} transport more rapidly and to a greater extent than an equivalent dose 25 HCC. 1,25 DHCC was found to increase serum Ca^{2+} of rats on low Ca^{2+} , vitamin D-deficient diet, but to a lesser degree than a similar dose of 25 HCC (Omdahl, et al, 1971b). The apparent short half life in vivo of 1,25 DHCC and the route of administration were the reasons given for the low antirachitic activity of 1,25 DHCC when compared to 25 HCC.

(5) Other metabolites

A metabolite of vitamin D_3 was isolated from plasma of pigs given large doses of vitamin D_3 . It was initially identified as 21,25 dihydroxycholecalciferol (Suda, DeLuca, Schnoes, Ponchon, Tanaka and Holick, 1970b). At a meeting of the International Congress of Endocrinology, Holick and DeLuca (1972) presented evidence that the compound was in fact 24,25 dihydroxy CC. Another compound was also isolated and identified as 25,26 dihydrocholecalciferol (Suda, DeLuca, Schnoes, Tanaka and Holick, 1970c) (see fig.I). The metabolite 21(24?)25 DHCC was only half as active as vitamin D_3 and 1/3 as active as 25 HCC in curing rickets in rats and in increasing intestinal Ca^{2+} transport

but was found to be more active than CC in bone mineral mobilization (Suda et al, 1970b).

The metabolite 25,26 DHCC according to Suda et al, (1970c) has some activity in intestinal Ca^{2+} transport, but was virtually inactive in the curing of rickets and in the mobilization of bone mineral in rats.

25 hydroxydihydrotachysterol has been synthesized by Suda, DeLuca, Schnoes and Hallick (1970a) and Suda, Hallick, DeLuca and Schnoes (1970d). Also Hallick and DeLuca (1971) have reported the biosynthesis of 25 hydroxydihydrotachysterol₃ in vivo and in vitro in rats, this being confirmed by U.V. spectrum and cochromatography. It was found to have weak antirachitic activity, but was a potent bone mobilization agent. It was also found to be more effective than dihydrotachysterol (DHT_3) in both capacities. It was more effective than DHT_3 in increasing intestinal Ca^{2+} transport and bone mobilization in thyroparathyroidectomised (TPTX) rats. It may be useful in the treatment of hypoparathyroidism and other similar bone diseases.

(6) The intracellular location of vitamin D and metabolites

Here again it is only with the advent of high specific activity of radioactive vitamin D and the use of a physiological dose, that the real pattern of distribution has been determined. Thus the work of early experimenters is only of historical importance.

Stohs and DeLuca (1967) found that with a dose level of 10 I.U. the radioactivity of the intestinal mucosa of chick was primarily in the curde nuclear fraction with little appearing in the mitochondria and microsomes. They found 57.6% of the radioactivity in

the nuclear debris fraction, but on purification using citric acid or Triton x 100, only 12% could be accounted for in the nuclear deoxyribonucleoprotein. It was found that the outer coats of the nuclei as well as the radioactivity were removed by these treatments. When a large dose of unlabelled vitamin D was given prior to a small dose of radioactive vitamin D, the incorporation of the radioactivity into the pure nuclei was greatly reduced. Stohs and DeLuca (1967) have estimated there are about 2000 functional sites for vitamin D metabolite per cell nucleus. After an oral, intracardial and intraperitoneal doses of generally labelled [^3H] vitamin D_3 , [$1,2^3\text{H}$] vitamin D_3 and [^{14}C] vitamin D_3 to rats, the selective localization of vitamin D was not found in the nuclear fraction of the kidney or liver, but was located in the intestinal nuclear fraction. The maximal amount of radioactivity in the nucleus was found within 3 hours of an intracardial injection of 15 or 50 I.U. of vitamin D. Only 0.4 I.U. of a dose of 15 I.U. of [^3H] vitamin D_3 administered intracardially was found in the intestinal mucosa; 75% of this was located in the nucleus. Haussler, Myrtle and Norman (1968) calculated this amount to be equivalent to 3,800 to 5,100 molecules of vitamin D and/or metabolites per mucosal cell. This is in agreement with the results of Stohs and DeLuca (1967). Similarly other workers have found the nuclei of the intestine contained radioactivity after the administration of radioactive vitamin D_3 (Haussler and Norman, 1969; Myrtle, Haussler and Norman, 1970; Cousins and DeLuca, 1970; Haussler et al, 1970a; Chen, Weber and DeLuca, 1970; Gray and DeLuca, 1971; Lawson et al, 1971b). Bone nuclei were also shown to contain 1,25 DHCC

(Lawson et al, 1971b; Weber, Pons and Kodicek, 1971). The supernatant of bone and intestinal mucosa cells were also found to contain the most polar metabolite (peak V) (Gray and DeLuca, 1971; Lawson et al, 1971a; Weber et al, 1971). However the actual site of radioactivity in the nuclei has not yet been resolved. There have been a number of reports that the radioactivity of 1,25 DHCC is associated with the chromatin (Haussler and Norman, 1969; Myrtle et al, 1970; Haussler et al, 1970a) while other workers reported the radioactivity is in the nuclear membrane fraction (Stohs and DeLuca, 1967; Chen et al, 1970). The controversy arises from the different methods used for the isolation and purification of the chromatin fractions.

Oku, Moriuchi and Hosoya (1971) and Hosoya and Oku (1971) reported the binding of vitamin D, 25 HCC and 1,25 DHCC to a specific soluble protein which was isolated from rat intestinal mucosa nuclear and cytoplasmic fractions. The nuclear fraction contained DNA and a small amount of RNA, while the cytoplasmic fraction contained little DNA. The binding to these proteins was unaffected by cholesterol, 7 dehydrocholesterol, estradiol or testosterone. It is now thought that steroids are bound first to a cytoplasmic protein before this complex can enter the nuclei.

(7) Sites of metabolism of the vitamin D

Horsting and DeLuca (1969a and b) reported that the metabolism of vitamin D in hepatectomised rats was not evident, indicating the liver as a possible major site of conversion of CC to 25 HCC. They also studied the metabolism of [^3H] vitamin D₃ in the perfused rat liver and in rat liver homogenate. In both these systems,

vitamin D_3 was converted to 25 HCC. The reaction in the homogenate was shown to require NADPH and the conversion was inhibited by high levels of peroxidase inhibitor. The production of 25 HCC in liver homogenate proceeded for 2 hours steadily and then decreased, which may indicate a feed-back mechanism. Ponchon and DeLuca, (1969b) Avioli et al, (1967) and Mawer et al, (1969) have shown that after an intravenous injection of [3H] vitamin D_3 , the disappearance of the radioactivity from the plasma was interrupted by a transient rebound (within 2-4 hours), due to a release of radioactivity from the liver to the blood. The rebound of radioactivity into the plasma coincides with the appearance of 25 HCC in the plasma and tissues. In hepatectomized rats, 25 HCC could be detected but was slow in appearing, whereas in the controls more than 15% conversion had occurred in 4 hours after the dose was administered.

In another experiment confirming the liver as the active site of conversion of vitamin D_3 to 25 HCC, the liver was isolated from the circulation system of rats. Almost all the [$1,2^3H$] vitamin D_3 remained unchanged (Ponchon, Kennan and DeLuca, 1969). The higher requirement for vitamin D observed in hepatic insufficiencies also lends support to this hypothesis. This concurs with what Horsting and DeLuca (1969b) had discovered about the liver homogenate hydroxylation of radioactive vitamin D_3 . Horsting and DeLuca (1970) showed that when the homogenate was prepared from rats predosed with vitamin D_3 , there was a dramatic fall in the rate of hydroxylation of radioactive vitamin D_3 . Also rats predosed with vitamin D_3 showed a lower rate of in vivo production of 25 HCC. In vitro studies showed

that low levels of 25 HCC added to liver homogenates strongly inhibited the hydroxylation of vitamin D₃, while 25 hydroxy - dihydrotachysterol (25 HDHT) did not inhibit this production. Ponchon and DeLuca (1970) reported that the production and degradation of 25 HCC was faster in chickens than rats due to a shorter lag time. This, as well as earlier data, suggests that there is a strong feedback control on 25 HCC production in liver cells which controls the level of circulating active form of vitamin D₃.

Hallick and DeLuca (1971) found that 25 HDHT was synthesized by liver homogenate. When 0.65 or 65 nmoles of [1,2-³H] dihydrotachysterol₃ (DHT₃) was given to rats, the serum contained 45% of the radioactivity in the form of 25 HDHT₃, 4 hours after the injection. A more polar metabolite than 25 HDHT represented 29% of the serum radioactivity 24 hours after a 0.65 nmole dose of DHT.

Hahn, Haddad, Birge and Avioli (1970) have reported the increased metabolism of vitamin D induced by phenobarbital in adult human's livers. The rate of conversion of [³H]vitamin D₃ to 25 HCC by the microsomes was increased. An in vitro incubation of [³H]vitamin D with liver microsomes from rats given phenobarbital (50 mg/kg/day for 4 days) and from control rats, showed a marked increase in the rate of [³H]vitamin D₃ conversion by the phenobarbital treated microsomes as compared with the control microsomes. It was suggested that there may be a link between the hepatic conversion of vitamin D and certain other steroid hormones.

Within 2 hours of an intravenous injection of [26,27-³H]25 HCC, it had been converted to peak V (1,25 DHCC) and a more polar

metabolite peak VI, which was found only in the duodenal part of the small intestine. Cousins and DeLuca (1970) and Cousins, DeLuca, Suda, Chen and Tanaka (1970) also suggested that $[1,2H^3]$ and $[26,27^3H]$ 25 HCC was converted in vivo by the intestinal mucosa to 1,25 DHCC and peak VI. Cousins, DeLuca and Gray (1970) studied the metabolism of 25 HCC in bone, intestinal mucosal cells, nuclei, kidney, liver and plasma of vitamin D-deficient rats in vivo. They thought that within 7.5 minutes the intestinal nuclei had converted 25 HCC into 2 metabolites, peak VI and V (1,25 DHCC). In bone, the peak VI intermediate was not detected and the quantity of peak V increased with time. They also reported a substantial metabolism of 25 HCC in the liver and kidney. A low circulating level of these metabolites is maintained in the plasma. Fraser and Kodicek (1970) and Lawson, Fraser, Kodicek, Morris and Williams (1971a) were the first to report the biosynthesis of 1,25 DHCC by the kidney. They found in nephrectomized rats that no 1,25 DHCC was formed. This was subsequently confirmed by Gray, Boyle and DeLuca (1971) and Norman et al (1971). Lawson et al (1971a) incubated $[26^{14}C][1^3H]$ 25 HCC with kidney homogenate in an original $^3H_3:^{14}C$ ratio of 2.5 : 1. Fifteen percent of 2 μ M 25 HCC was converted to 1,25 DHCC by this homogenate from chicks in 2 hours. This was confirmed by Gray et al (1971) and Norman et al (1971). In an in vivo experiment nephrectomy was not found to impair synthesis of $[1^3H]$ $[26^{14}C]$ 25 HCC, but did affect formation of 1,25 DHCC (Norman et al, 1971; Hill, Van Der Berg and Mawer, 1971). This 1,25 DHCC is possibly then distributed to target tissues by way of blood. It has been suggested

that it should be called a hormone because it is secreted from the kidney and circulates to the target organ.

These results thus demonstrate that the liver is the major site of conversion of CC to 25 HCC and that the kidney is the site of conversion at 25 HCC to 1,25 DHCC.

(8) The effect of actinomycin D upon the action of vitamin D on calcium metabolism.

In the last few years the action of vitamin D on the transcription of DNA and protein synthesis has been studied. This transcription may be important in translation of the message and is probably involved in production of new protein and enzymes.

Eisenstein and Passavoy (1964) observed the inhibition of DNA transcription into RNA by the antibiotic actinomycin D and the simultaneous blockage of the hypercalcemic response usually obtained with a large dose of vitamin D. Zull, Czarnowska-Misztal and DeLuca (1965 ; 1966) showed, also, that actinomycin given 1 hour before a dose of vitamin D completely inhibited the usual effect of the vitamin on increasing serum Ca^{2+} and increasing Ca^{2+} transport in vitro or in vivo. In chicks, Norman (1965 ; 1966a) and Norman, Haussler, Adams, Myrtle, Roberts and Hibberd (1969) demonstrated the blocking action of actinomycin D on increased Ca^{2+} absorption. The inclusion of puromycin in the diet of rachitic rats prevented the usual manifestation of vitamin D on Ca^{2+} absorption (Schachter and Kowarski, 1965). Zull et al, (1966) reported that not only actinomycin but also fluororotic acid and puromycin inhibited the action of vitamin D. The effect caused by actinomycin was not due to the inhibition of

distribution of vitamin D, the inhibition of the PTH synthesis, or to general toxicity or cell necrosis. A series of important experiments showed that if vitamin D was given to an animal 4-8 hours prior to this actinomycin D there was no inhibition of the vitamin D enhancement of Ca^{2+} transport. Thus actinomycin D was considered to inhibit a step in the physiological expression of the vitamin.

Both Zull et al, (1966) and Norman (1966a) found that actinomycin D did not alter the tissue distribution of vitamin D after doses of 2,000 I.U., or 350 I.U. of [^3H]vitamin D in rachitic rats and chicks respectively.

The information so far does not unequivocally establish that vitamin D or metabolites acts primarily to initiate the synthesis of protein or the utilization of genetic information. However, Norman (1966b) showed that there was a three fold increase in the incorporation of [^3H]uridine into RNA after a 5,000 I.U. dose of vitamin D was given to rachitic chicks.

This process of incorporation was inhibited by actinomycin and o.p. D.D.D, which suggests that the primary action of vitamin D is upon the initiation of RNA synthesis in the intestinal mucosa. In a similar experiment where 2,000 I.U. of vitamin D was injected intraperitoneally, a stimulation of incorporation of [^3H]orotic acid into nuclear RNA was observed 3 hours after injection of the vitamin. Stohs and DeLuca (1967) showed that vitamin D caused an increase in incorporation of [^3H]orotic acid into nuclear RNA and this was blocked by a prior administration of actinomycin D. RNA was maximally labelled after 3 hours but decreased to an insignificant level within 8 hours.

With a dose of 10 I.U. vitamin D, RNA labelling response was maximal at 5-8 hours. In addition the template activity for DNA-dependent RNA synthesis was significantly increased by the administration of vitamin D to vitamin D-deficient rats (Hallick and DeLuca, 1969)

DeLuca (1969a) reported that the action of vitamin D and 25 HCC on bone and intestine was blocked by prior administration of actinomycin D. He also reported the stimulation of RNA labelling by [^3H] orotic acid within minutes after intravenous administration of 25 HCC. The results suggest that the site of subcellular action of 25 HCC and 1,25 DHCC is the nucleus. DeLuca (1971) postulated that the new proteins synthesised in the presence of 25 HCC and 1,25 DHCC acted at the brush borders to enhance Ca^{2+} uptake. When Lawson, Wilson, Barker and Kodicek (1969) isolated chick intestinal nuclei practically free of contaminating organelles and whole cells, the proportions of RNA, DNA, and protein of the isolated nuclei were unaffected by vitamin D status of the birds. The incorporation of [^3H] orotic acid into rapidly labelled intestinal nuclear RNA, after a 1 minute pulse of orotic acid, was increased within 10 minutes when the vitamin D-deficient chick were given a 125 μg dose of cholecalciferol. There was no stimulation of DNA dependent RNA polymerase activity of the isolated nuclei from birds treated with cholecalciferol. This stimulation of nuclei in vitro by vitamin D_3 itself is difficult to understand.

Kodicek et al, (1970) has shown that administration of vitamin D_3 in vivo is converted to its dihydroxy- form before transference to the intestinal nuclei. Adams and Norman (1970) provided

evidence that the enhancement of Ca^{2+} transport brought about by vitamin D was achieved indirectly through RNA and protein synthesis, rather than direct participation of the vitamin. The increased Ca^{2+} flux due to vitamin D_3 administration was found to require 40-50 hours for optimal development and was blocked by prior actinomycin treatment. Vitamin D_3 action on $[5^3\text{H}]$ uridine incorporation into rat intestinal mucosal RNA was studied by Iitoya, Moriuchi, Tahase and Hosoya (1971). The specific activity of RNA in nuclei, mitochondria and supernatant increased 2 hours after uridine administration, but it decreased in the microsomes. The administration of 2,000 I.U. vitamin D_3 before uridine increased the specific activity of RNA in the nuclear and supernatant fractions. The Mg^{2+} -dependent RNA polymerase activity was not increased 1 hour after vitamin D_3 administration but was decreased in the period from 3 to 16 hours after vitamin D_3 treatment. This decrease corresponds to the decrease in incorporation of $[^3\text{H}]$ uridine into RNA.

In 1971 several experiments were done to verify that the active form of vitamin D present in the nucleus is 1,25 DHCC (Norman et al, 1971; Lawson et al, 1971a and b; Gray et al, 1971). Prior administration of either cycloheximide or actinomycin D to vitamin D-deficient rats blocked the conversion of 25 HCC to 1,25 DHCC in the kidney (Haussler et al, 1970; Gray and DeLuca, 1971; Tanaka and DeLuca, 1971; Omdahl, et al, 1971a) and blocked the response of Ca^{2+} transport to 25 HCC (Omdahl et al, 1971a). The intestinal Ca^{2+} transport response due to 1,25 DHCC was insensitive to the antibiotic (Tanaka, DeLuca, Omdahl and Holick, 1971). These results suggest that 1,25 DHCC, or a further metabolite, is the active form of the vitamin

in promoting increased intestinal Ca^{2+} absorption. Since actinomycin prevents the enhancement of Ca^{2+} transport, usually brought about by 25 HCC, the enzyme for converting 25 HCC to 1,25 DHCC is either one of rapid turnover, or 25 HCC itself brings about the formation of the 1 hydroxylase.

It has been demonstrated that 1,25 DHCC arrives in the intestine 1 hour after a dose of CC, or 30 minutes after dose of 25 HCC given intracardially (Lawson et al, 1971a) while Ca^{2+} transport in the intestine is enhanced 5 hours after the dose. Presumably the 4 hour delay of increased Ca^{2+} transport is due to the time required to synthesise new proteins.

(9) The regulation of synthesis of 1,25 dihydroxycholecalciferol and 24,25 dihydroxycholecalciferol

Boyle, Gray and DeLuca (1971) have reported that 12 hours after the administration of 325 pmoles [^3H] 25 HCC to rats, [^3H] 1,25 DHCC accumulated in several tissues to an extent that varied with the dietary Ca^{2+} content. As the dietary and serum Ca^{2+} concentration increased the amount of 1,25 DHCC diminished and the concentration of 24,25 DHCC increased. Almost complete repression of 1,25 DHCC formation in vitamin D-deficient rats occurred when the diet contained 3% Ca^{2+} and 20% lactose. These results suggest that the production of 1,25 DHCC is responsible for the adaptation of Ca^{2+} absorption to a low dietary concentration of Ca^{2+} , that 24,25 DHCC could be involved in the kidney excretion of Ca^{2+} , and that Ca^{2+} concentration may regulate 1 hydroxylase activity in the kidney. The plasma concentration of Ca^{2+} is in turn regulated by the PTH and CT and cellular Ca^{2+} levels by the

plasma Ca^{2+} . In growing animals, 1,25 DHCC is possibly produced in preference to 24,25 DHCC thus enhancing Ca^{2+} absorption.

(10) Vitamin D induced synthesis of proteins

Wasserman and Taylor (1966) have shown an increase in calcium binding protein (CaBP) in the intestinal mucosa, 8 hours after a dose of the vitamin. The formation of this CaBP reached a maximum at about 72 hours in the chick intestine. This appears to indicate that new protein synthesis is occurring at the same time that RNA synthesis and Ca^{2+} absorption are being stimulated by vitamin D. The topic of the CaBP will be dealt with in a later section.

Urban and Schedl (1969c) have suggested that vitamin D (12.5 μg , 50 I.U.) given to rats (50-70g) significantly increased the duodenal growth of the mucosa for 4 days, but after this time there was no further growth. The effect on ileal mucosa was not significant. The turnover time of mucosal cells was 30-40 hours. Vitamin D did not increase water content in the tissue.

Martin, Melancon and DeLuca (1969) reported that a Ca^{2+} -dependent ATPase in the brush borders of rat and chick small intestine was markedly increased after vitamin D administration to vitamin D-deficient and Mg^{2+} -deficient rats. This enzyme was suggested to be concerned with the vitamin D-directed absorption of Ca^{2+} in the intestine. The initial Ca^{2+} uptake across the brush borders surface was found to be an oxygen dependent, vitamin D stimulated process which was carrier mediated.

According to Melancon and DeLuca (1970) and Melancon (1970), vitamin D, by its effect on protein synthesis, caused the increase in

active transport of Ca^{2+} in intestine of vitamin D-deficient animals. This Ca^{2+} transport process could be located at the brush border region of the cell. The membrane-associated ATPases and phosphatases were investigated by a number of workers. Vitamin D was found to give rise to an increased amount of Ca^{2+} -dependent ATPase (Martin, Melancon and DeLuca, 1969; Melancon, 1970; Melancon and DeLuca, 1970; Holdsworth, 1970; Haussler, Nagode and Rasmussen, 1970b; Norman, Mircheff, Adams and Spielvogel, 1970; Taylor and Wasserman, 1970b), alkaline phosphatase (Holdsworth, 1970; Norman et al, 1970; Haussler et al, 1970b; Taylor and Wasserman, 1970b), pyrophosphatase (Holdsworth, 1970) and leucine aminopeptidase (Taylor and Wasserman, 1970b) all located in the brush borders. The time course of increase of Ca^{2+} -dependent ATPase and alkaline phosphatase activity after oral and intravenous vitamin D_3 and 25 HCC administration compared well with the time course of appearance of Ca^{2+} absorption in vivo and in vitro intestinal segments (Melancon, 1970; Melancon and DeLuca, 1970; Norman et al, 1970). A two fold increase in Ca^{2+} -ATPase (Holdsworth, 1970; Norman et al, 1970), alkaline phosphatase (Holdsworth, 1970; Norman et al, 1970) and pyrophosphatase (Holdsworth, 1970) was observed 16 hours after a 400-500 I.U. dose of vitamin D_3 . The Ca^{2+} -ATPase increase in activity was not observed before 19.5 hours after vitamin D administration according to Melancon (1970). After the administration of 100 I.U. vitamin D_3 to rachitic chicks, CaBP and alkaline phosphatase (APase) were first to show an increase (24 hours), followed by leucine amino peptidase (LAPase) (72 hours), with no increase in Ca^{2+} -ATPase at the

longest time period examined (72 hours). A higher dosage of vitamin D, 500 I.U., was needed before an increase in Ca^{2+} -ATPase was observed. The low Ca^{2+} diet (0.1%) was found to increase brush border LAPase activity after 3 days, while there was no change in APase or Ca^{2+} -ATPase activities after 3 to 8 days (Taylor and Wasserman, 1970b).

This Ca^{2+} -ATPase activity was stimulated by Mg^{2+} , Sr^{2+} , Ba^{2+} and K^{+} to a lesser degree than by Ca^{2+} , while Mn^{2+} inhibited its activity (Melancon and DeLuca, 1970; Melancon, 1970). Holdsworth (1970) reported that both the ATPase and APase activities required Mg^{2+} and were enhanced by Ca^{2+} addition. He also observed that the ATPase, APase and pyrophosphatase activities were inhibited by phenylalanine (20mM). Holdsworth (1970) concluded these three activities were due to the same enzyme, while Haussler et al (1970b) found that L-phenylalanine, theophylline and diphosphonate inhibited Ca^{2+} - and Mg^{2+} -stimulated ATPase and APase activities. This suggests that Ca^{2+} -ATPase and APase were the same enzyme. The inhibition of phosphatase by phenylalanine was found not to inhibit Ca^{2+} translocation in the everted sac of the chick intestine (Holdsworth, 1970). Prior administration of cycloheximide or actinomycin D to vitamin D was found to inhibit alkaline phosphatase increased activity in chicks (Holdsworth, 1970; Norman et al, 1970; Haussler et al, 1970b). This suggests that the vitamin induces the de novo synthesis of this enzyme (Norman et al, 1970). These increases in enzyme activities may be due to the growth promoting action of the vitamin suggested by Urban and Schedl, (1969c), but their role in the Ca^{2+} translocation process has yet to be proved.

(11) The existence of a calcium binding protein present in the cell after a dose of vitamin D.

There has been specific attention given to the fact that vitamin D required 8-16 hours before the absorptive mechanism is partially restored in the rachitic animals. The possible sequence of events in the lag period are (a) the transit of the vitamin to the liver with conversion to 25 HCC, (b) the transformation of 25 HCC in the kidney to 1,25 DHCC, (c) the transfer of 1,25 DHCC to the intestinal site via the blood (d) the direct or indirect interaction of a metabolite with the genetic information, as shown by the increased incorporation of RNA precursors into RNA and the inhibitory effect of actinomycin on the vitamin's response and (e) the synthesis of protein that may be involved in Ca^{2+} translocation.

Wasserman and Taylor (1963) and Taylor and Wasserman (1965) described a vitamin D_3 dependent factor which influenced Ca^{2+} binding by homogenates of chick intestinal mucosa, and which could not be induced by the addition of vitamin D to the homogenate. This Ca^{2+} binding factor was found to be a protein. It was present in the supernatant fluid after removal of the heavy microsomes (Wasserman and Taylor, 1966; Taylor and Wasserman, 1967). This protein is subsequently referred to as the calcium binding protein (CaBP). These observations in chicks were not confirmed in an early report by DeLuca (1967) using rats because he was unable to observe its presence in the homogenate and cytoplasm of intestines from vitamin D-repleted rats at an early period after vitamin D administration. It was subsequently shown that CaBP was present in small amounts in the small intestine (Harmeyer and DeLuca, 1969). The failure possibly was due

to the diet being fed to the rats at the time of isolation and also upon the age of the rats. However Kallfelz, Taylor and Wasserman (1967) were able to detect the presence of the CaBP in the homogenate of duodenal mucosa of rat intestine 72 hours after a 500 I.U. dose of vitamin D₂ or vitamin D₃ had been given. The CaBP was found in the protein portion of the supernatant after passage through a Sephadex G25 column.

Harmeyer and DeLuca (1969) reported that CaBP formation and the Ca²⁺ absorption increase occurred at different times after vitamin D administration in chicks and rat intestines. The lag phase of Ca²⁺ absorption after an oral 500 I.U. dose of vitamin D in chicks was about 10-16 hours depending on Ca²⁺ concentration in the diet. However, the lag phase of CaBP formation was 19-20 hours. The slopes of the two parameters between 20 and 100 hours after vitamin D administration were also different. Lowering the Ca²⁺ concentration of the diet reduced the lag phase of Ca²⁺ absorption from 16 to 10 hours after vitamin D, but had no effect on the lag phase of CaBP formation. Thus they concluded that Ca²⁺ absorption and CaBP were not directly related even though CaBP was present at initiation of Ca²⁺ absorption. These results could have differed from Wasserman's group because of the method of detection of CaBP at low levels.

The vitamin D-dependent CaBP has been shown to be present in the intestinal mucosa of rat (Kallfelz, Taylor and Wasserman, 1967; Taylor, Wasserman and Jowsey, 1968; Schachter, 1969; Moriuchi, Ooizumi and Hosoya, 1969; Drescher and DeLuca, 1971a and b), monkey (Wasserman and Taylor, 1971), dog (Taylor, Wasserman and Jowsey, 1968), hamsters

(Schachter, Kowarski and Reid, 1967), chickens (Wasserman and Taylor, 1966), cow (Fullmer and Wasserman, 1972) and human (Hitchman and Harrison, 1972; Piazzola, Schleyer and Franz, 1971; Hitchman, Finlay and Harrison, 1971).

This protein was present in all the segments of the chick small intestine (Taylor and Wasserman, 1967), the colon (Taylor and Wasserman, 1969a ; 1970a), in the kidney (Taylor and Wasserman, 1967, 1969b, 1971) and the shell gland of laying hens (Corradino, Wasserman, Pubols and Chang, 1968). The concentration of CaBP was shown to decrease from the duodenum to the ileum and colon in the vitamin D-replete chicks (Taylor and Wasserman, 1967 ; 1969a), new World monkeys (Wasserman and Taylor, 1971) and hamsters (Schachter, Kowarski and Reid, 1967).

The time course of CaBP formation by intestinal tissue from rachitic chick, after vitamin D₃ administration, paralleled the enhanced Ca²⁺ absorption and varied with the dose of vitamin D₃, (Wasserman and Taylor, 1966; Taylor and Wasserman, 1967; Ebel, Taylor and Wasserman, 1969). The rate of decay of both the Ca²⁺ binding protein and the vitamin-stimulated Ca²⁺ absorption was about the same in chicks that have been given a dose of vitamin D and then allowed to become rachitic. It has been observed that the CaBP content of the intestine decreases with age (Wasserman and Taylor, 1968), it was greater in laying hens than non-layers (Wasserman and Taylor, 1968; Hurwitz and Bar, 1969b) and increased in chick when adapted to a low Ca²⁺ intake. During adaptation studies on chicks the rate of Ca²⁺ absorption under steady state conditions was found to be highly correlated with the CaBP

concentration (Morrissey and Wasserman, 1970). Further, under conditions of varying Ca^{2+} and PO_4^{3-} intakes by chicks, the correlation coefficient between the estimated rate of $^{45}\text{Ca}^{2+}$ absorption and intestinal CaBP levels was very high at about 0.99. A low PO_4^{3-} diet with normal or higher than normal Ca^{2+} , increased both the capacity of the intestine to absorb Ca^{2+} and the concentration of CaBP in the intestinal mucosa. Morrissey and Wasserman (1970) suggested the stimulus for adaptation may be multiple, and that factors related to bone mineralization were undoubtedly involved.

In contrast, Krawitt and Kunin (1971) reported that the level of intestinal CaBP activity was the same in rats raised on low and high PO_4^{3-} diets. With vitamin D administration, the induction of increased binding activity was of the same magnitude in both groups. Thus CaBP synthesis appears to be independent of dietary PO_4^{3-} deficiency and of the presence of rickets.

Vitamin D_2 which has less antirachitic activity in the chick than vitamin D_3 was observed to be 10 times less effective in promoting the synthesis of calcium binding protein (Wasserman and Taylor, 1966). Corradino and Wasserman (1970a and b; 1971a and b) reported the induction of CaBP in chick embryonic intestine membranes in organ culture by the addition of vitamin D_3 to the culture medium. The detection of CaBP was accomplished by using a sensitive immunoassay. Dihydratachysterol series 2 and 25 hydroxycholecalciferol were found to be equally or more effective than vitamin D_3 in inducing CaBP formation, while other sterols were found to be ineffective (Corradino and Wasserman, 1971b). Accompanying induction of this protein there was enhanced $^{45}\text{Ca}^{2+}$ uptake

by the intestine (Corradino and Wasserman, 1971a and b). In a later report, Corradino and Wasserman (1972) stated that as little as 6.5 pmoles/ml of either vitamin D₃ or 25 HCC in culture medium would induce detectable amounts of CaBP. With 10 times this amount, ⁴⁵Ca²⁺ uptake was enhanced. However as little as 6.5 femtomoles/ml of 1,25 DHCC in the culture medium were able to induce CaBP synthesis. At 6.5 pmoles/ml of 1,25 DHCC were 10 times more potent than vitamin D₃ and the 25 HCC was twice as potent as vitamin D₃ in the induction of CaBP. This amount of 1,25 DHCC was twice as potent as vitamin D₃ in the stimulation of Ca²⁺ uptake. At this level 25 HCC was shown not to have an effect on Ca²⁺ uptake.

These observations indicate that in this system, the transformation of vitamin D₃ to an active form by a tissue remote from the intestine is not required for at least one aspect of the biological action of vitamin D (Corradino and Wasserman, 1970a). This is the first direct in vitro physiological effect of vitamin D₃ on the Ca²⁺ absorptive mechanism of the intestine found. Maximum Ca²⁺ absorption and CaBP formation occurred 72 hours after vitamin D administration and then declined.

When Sr²⁺ replaces Ca²⁺ in the diet to the extent of molar equivalents (0.2% Ca²⁺), Sr²⁺ causes a depletion of CaBP (Taylor and Wasserman, 1969b; Corradino and Wasserman, 1970a; Corradino, Ebel, Craig, Taylor and Wasserman, 1971a). Rapid restoration of CaBP levels and Ca²⁺ absorptive efficiency follows, when Sr²⁺-inhibited chicks are placed on normal diet (Corradino, Ebel, Craig, Taylor and Wasserman, 1971b).

The CaBP was not detected in bone, blood, liver, or pancreas by immunoassay (Wasserman, Corradino and Taylor, 1969; Taylor and Wasserman, 1970a) but it has been localized in the small intestine, colon and kidney (Taylor and Wasserman, 1970a). By fluorescent antibody techniques, the CaBP was located in the goblet cells and microvillar region of the intestinal mucosa from normal and vitamin D treated chicks (Taylor and Wasserman, 1970a).

In the presence of vitamin D or its metabolites, the formation or synthesis of CaBP in the embryonic chick intestine was dependent on the concentration of Ca^{2+} in the medium, starting at 0.15 mM- Ca^{2+} , and reached a maximum between 0.31 and 0.62mM added Ca^{2+} and declined at 1.25mM and was again detected above 2.5mM added Ca^{2+} in the medium. (Corradino and Wasserman, 1971a).

Embryonic chicken intestine in culture did not increase CaBP production when some other divalent ions were in the medium (Be^{2+} , Mg^{2+} , Sr^{2+} or Ba^{2+}) at 0.31mM (Corradino and Wasserman, 1971a). The specificity and biphasic nature of the stimulatory-inhibitory action of Ca^{2+} concentration on CaBP synthesis may have physiological implications in the regulation of intestinal Ca^{2+} absorption. They suggested that CaBP synthesis or Ca^{2+} absorption by the intestine in vivo may be regulated by the ambient Ca^{2+} concentration at some critical intracellular site.

Taylor and Wasserman (1971) have shown that unlike the embryonic intestine which contains none or very little CaBP (Taylor and Wasserman, 1969a ; 1972), both intermediate and definite kidney of developing chick contains CaBP. It was present at 10 days in the

embryo kidney and the concentration remained constant until day 17 when it started to decrease. The CaBP in the kidney remained present while the chick was on a rachitogenic diet for 6 weeks, but it did decrease with age and correlated with the rate of decrease in serum Ca^{2+} concentration. Intestinal mucosa contained CaBP from the day of hatching up to end of first week on the rachitogenic diet. The presence and absence of CaBP in intestine and kidney from chicks raised on a rachitogenic diet, was shown to reflect the relative cell turnover of the two tissues (Taylor and Wasserman, 1972). The presence of CaBP in kidneys of otherwise rachitic chicks may explain the difficulties reported in demonstrating a vitamin D effect on Ca^{2+} reabsorption of Ca^{2+} in the kidney.

MacGregor, Hamilton and Cohn (1970) investigated the effect of vitamin D_3 on de novo biosynthesis of the CaBP in chick intestinal mucosa and the temporal relationship between such synthesis and the stimulation of intestinal Ca^{2+} transport by the vitamin. When a dose of 20 I.U. of vitamin D_3 was given to either vitamin D-deficient or partially vitamin D-deficient chicks, the rate of biosynthesis of CaBP was evaluated by the amount of incorporation into isolated CaBP of [^3H]leucine which was given intraperitoneally three hours before killing. In rachitic chicks the incorporation of leucine into isolated CaBP rose from an undetectable amount to a substantial rate in 24 hours. In partially vitamin D-deficient chicks a 3-4 times increase in incorporation rate was seen between zero and 12 hours. Incorporation of isotope into total mucosal protein was unaffected at the 20 I.U. dose level in either type of chick, but was stimulated 50% after 24 hours by

treatment of rachitic chick with high doses (500 I.U.). This indicates that large doses of vitamin may elicit rapid non-specific stimulatory effect on growth and metabolism, in contrast to the apparently specific effect on Ca^{2+} transport of small doses. This may spread light on the increased enzyme activity in the microvilli region. Simultaneous measurement of the effect of vitamin D on Ca^{2+} transport and rate of CaBP biosynthesis in partially vitamin D-deficient chicks, indicated that the rate of CaBP biosynthesis was stimulated by vitamin D several hours before an effect on transport could be detected. Nine hours after the administration of 20 I.U. vitamin D_3 , [^3H] leucine incorporation into pure CaBP was stimulated two or three fold, several hours before any effect on intestinal Ca^{2+} transport was noted. The inductions of Ca^{2+} transport and the appearance of the CaBP was observed to occur simultaneously. This data supports the hypothesis that vitamin D acts at the genetic level and also suggests that CaBP may play a primary role in vitamin D mediation of Ca^{2+} transport. An effect at the genetic level may account for the increase in some enzyme activities after intake of vitamin D (Taylor and Wasserman, 1970b).

The CaBP was shown to have the property of forming a tight complex with Ca^{2+} (Wasserman and Taylor, 1966). It has been isolated in high purity from chick intestinal mucosa by gel filtration and preparative electrophoresis (Wasserman, Corradino and Taylor, 1968). It possesses a molecular weight between 24,000 - 28,000, has a Ca^{2+} formation constant of $2.60 \times 10^5 \text{M}^{-1}$, has the ability to bind 1 mole of Ca^{2+} per mole of protein (Wasserman et al, 1968) and is heat stable

to 70°C for 10 minutes. Recently Wasserman and Corradino (1971) reported the CaBP to possess a high affinity constant of about 10^6 M^{-1} . Chick Ca^{2+} binding protein contains large amounts of aspartic acid, glutamate; lysine and leucine (Wasserman et al, 1968). Alkaline earth cations bind to CaBP in the following order $\text{Ca}^{2+} > \text{Sr}^{2+} > \text{Ba}^{2+} > \text{Mg}^{2+}$, the rate of absorption of each of these alkaline metals follows the same relative order (Taylor and Wasserman, 1969b; Ingersoll and Wasserman, 1971). Since N-ethylmaleimide, iodoacetate and p-chloromercuribenzoate do not inhibit the binding reaction, sulphhydryl groups must not be directly associated with the binding sites (Ingersoll and Wasserman, 1971). The isoelectric point of the protein was found to be about pH 4.3 and the pH dependency of the binding reaction shows two maxima, one at about pH 6.3 and the other at about pH 9.2. There appears to be between 30-40% helicity associated with the protein. Its synthesis was inhibited by prior administration of an inhibitor of protein synthesis (Corradino and Wasserman, 1968; Taylor and Wasserman, 1969a). Lysolecithin, cetrinide, myristoylcholine form a complex with the protein and reduces its capacity to bind Ca^{2+} (Wasserman, 1970; Ingersoll and Wasserman, 1971). Substances without effect on binding properties of the protein include choline, acetylcholine, propionylcholine, taurocholate, deoxycholate and Tween 80 (Ingersoll and Wasserman, 1971). It was suggested that both the hydrophobic moiety and the quaternary ammonium functional groups were required for the inhibition of Ca^{2+} binding.

Moriuchi, Ooizumi and Hosoya (1969) reported that the CaBP

they isolated from rat intestinal mucosa, was Ca^{2+} specific and consisted of two components. The first component was increased by vitamin D_3 administration, suppressed with actinomycin D pre-treatment, and decreased by trypsin digestion (Moriuchi et al, 1969). On the other hand the second component was not influenced by vitamin D_3 , but increased by trypsin digestion. These studies were done at 4 and 16 hours after vitamin D administration. The second component could be a smaller fragment of the first. Drescher and DeLuca (1971a and b) and Moriuchi et al (1969) found that two binding proteins could be isolated from rat intestinal supernatant. Drescher and DeLuca (1971b) suggested one of these proteins as a precursor for the other, the precursor having a molecular weight of 13,000 and the binding protein a molecular weight 8,000 - 9,000.

Hitchman, Finlay and Harrison (1971) also reported two components in rat, pig and human mucosa that bound Ca^{2+} , one being of high molecular weight and the other with a molecular weight of about 12,000 - 13,000.

From evidence presented one can appreciate that the calcium binding protein is intimately involved in the translocation of Ca^{2+} across the intestinal epithelium. But conclusive information is not yet available to suggest exactly how it does this. It is not inconceivable that its site and/or sites of action correspond to those points of intestinal cell which, from physiological data, are most effected by the presence of vitamin D (Taylor and Wasserman, 1967). These authors suggested that this Ca^{2+} binding protein was involved in translocation and was used to shuttle Ca^{2+} . Another hypothesis,

Schachter et al (1967), suggests that vitamin D stimulated the synthesis of transport carriers which may include CaBP. The uptake of Ca^{2+} into the epithelial cells being brought about by the carrier at the mucosal surface and then moved along the activity gradient. Subsequently transfer out of the cell at the serosal surface occurs against activity gradients, and could result from a dissociation of the carrier Ca^{2+} complex in the membrane by the relatively high concentration of extracellular Na^{+} . Wasserman, Corradino and Taylor (1969) suggest that there could be three mechanisms in which this protein may be involved: (1) a diffusional facilitator (to enhance transfer of Ca^{2+} across the microvillar membrane), (2) intracellular carrier (facilitates movement of Ca^{2+} through the cytoplasm and actively extrudes Ca^{2+} from the cell at the site of the Ca^{2+} pump at the base of the cell) and (3) microvillar concentration (to concentrate Ca^{2+} at microvilli by acting analogous to a resin and then possibly being involved in cellular transport).

This thesis attempts to show a role for CaBP in Ca^{2+} transport in the intestine.

(12) Summary

The metabolism of vitamin D is now known to proceed by the conversion of the vitamin in the liver to the 25 hydroxylated form. This compound is then transported by the plasma to the kidney, where it is hydroxylated to form the 1,25 dihydroxy compound. The 1,25 dihydroxy compound is then transported by plasma to the target organs of the intestine and bone, where it may effect transcription of DNA, thus leading to the formation of proteins concerned in Ca^{2+} transport

e.g. CaBP, alkaline phosphatase, and Ca^{2+} -ATPase.

THE ACCUMULATION AND RELEASE OF CALCIUM BY MITOCHONDRIA

This subject has been recently reviewed by Lehninger, Carafoli and Rossi (1967) and more recently by Lehninger (1970).

One of the earliest reports on the subcellular distribution of metal ions was by Thiers and Vallee (1957). By fractionation of rat liver homogenate they showed that the mitochondria contained large quantities of Mg^{2+} and K^{+} and smaller amounts of Na^{+} and Ca^{2+} . Similar results were obtained in beef heart mitochondria by Wester (1965) using a sensitive method of gamma spectrometry. The Ca^{2+} concentration in the mitochondria was determined to be $8 \times 10^{-4}\text{M}$ or 10.8 nmoles per mg protein and the cytoplasm contained 5×10^{-5} to 10^{-6}M or 2.0 nmoles per mg protein for rat liver (Thiers and Vallee, 1957; Drahota, Carafoli, Rossi, Gamble and Lehninger, 1965). Bygrave, Reid and Spencer (1971) have shown that free Ca^{2+} bears a linear relationship to its concentration and to mitochondrial uptake.

Vasington and Murphy (1961 and 1962) did the first comprehensive study of Ca^{2+} uptake by mitochondria. They found that rat kidney mitochondria rapidly accumulated Ca^{2+} from a medium containing Mg^{2+} , respiratory substrate, ATP or ADP and inorganic phosphate (Pi). These mitochondria accumulated as much as 200 times the initial endogenous Ca^{2+} level. It was established that during massive loading: (a) Ca^{2+} uptake required electron transport from respiratory substrates, (b) Ca^{2+} uptake required the presence of ATP or ADP, Mg^{2+} and Pi, (c) no oxidative phosphorylation of ADP occurred when Ca^{2+} was accumulated, (d) Ca^{2+} uptake was inhibited by 2,4

dinitrophenol (2,4DNP) and other uncoupling agents, even without oxidative phosphorylation, (e) oligomycin did not inhibit Ca^{2+} uptake, (f) Ca^{2+} uptake was very rapid, maximal loading was complete in 10 minutes at 37°C and (g) neutral salts such as NaCl, KCl and LiCl promote Ca^{2+} uptake. Some other characteristics of massive loading of Ca^{2+} have been discovered. When Ca^{2+} was accumulated, Pi was also accumulated and the molar ratio $\text{Ca}^{2+}:\text{Pi}$ accumulated was about 1.67 (Rossi and Lehninger, 1963; Lehninger, Rossi and Greenawalt, 1963). The same components were required for Pi accumulation as for Ca^{2+} , and the same agents that inhibited Ca^{2+} uptake also inhibited Pi accumulation. Rossi and Lehninger (1963) and Brierly, Murer and Bachmann (1964) found that during Ca^{2+} and Pi accumulation in the absence of electron transport, the addition of Ca^{2+} greatly stimulated ATP hydrolysis by mitochondria. The molar ratio of Ca^{2+} accumulated to ATP hydrolysed was found to be only about 0.5. The uptake of Ca^{2+} and Pi were found to be stoichiometric with electron transport such that about 1.7 - 2.0 molecules Ca^{2+} and 1.0 molecule PO_4^{3-} were accumulated per pair of electrons traversing each of the three energy conserving sites of the respiratory chain (Rossi and Lehninger, 1963). During massive loading of mitochondria with Ca^{2+} and Pi, there was irreversible damage to oxidative phosphorylation and respiratory control mechanisms as well as morphological changes (Greenawalt, Rossi and Lehninger, 1964).

In limited loading of mitochondria with Ca^{2+} (less than 100 nmoles Ca^{2+}/mg protein), it was found that Ca^{2+} stimulated respiration of mitochondria in a stoichiometric and cyclic fashion so that the

amount of extra oxygen uptake caused by two calcium ions was the same as caused by one molecule of ADP (Chance 1963, 1965; Rossi and Lehninger, 1963). In the absence of Pi, ATP and Mg^{2+} , but in the presence of substrate, Ca^{2+} uptake stimulated respiration so long as a significant concentration of Ca^{2+} (above $1-2 \mu M$) remained free in the suspending medium, otherwise the respiration returned to the resting state (Drahota et al, 1965). The $Ca^{2+} : \sim$ accumulation ratio with different respiratory substrates revealed that about 1.7 - 2.0 molecules Ca^{2+} were accumulated per electron conserving site (Rossi and Lehninger, 1964). This agreed closely with results for massive loading experiments. Neither oligomycin nor atractyloside inhibited this respiration linked Ca^{2+} uptake in the absence of phosphate (Rossi and Lehninger 1964; Carafoli, Patriarca and Rossi, 1969). The capacity of mitochondria to accumulate Ca^{2+} in the absence of phosphate was very limited, since the $Ca^{2+} : \sim$ ratio remained stoichiometric at about 2.0 only up to Ca^{2+} loads not exceeding about 100 nmoles per mg protein (Chance, 1964). Neither endogenous ATP nor Pi appear to be required for Ca^{2+} uptake, since prior depletion of mitochondrial phosphate and ATP did not diminish the capacity (Rossi and Lehninger, 1964).

In the absence of ATP and Mg^{2+} , but in the presence of substrate, and with concentrations of Pi below 1mM, Pi was accumulated with Ca^{2+} during respiratory jump, and oxygen uptake returns normally to the resting state under limiting loading conditions (Rossi and Lehninger, 1964). However, under similar conditions, if the Pi concentration exceeds 2.0 mM, then respiration stimulated by Ca^{2+} may

continue indefinitely without returning to the resting state. Under these conditions less than 10% of the Ca^{2+} was accumulated (Rossi and Lehninger, 1964). This discharging effect of excess Pi was prevented by the addition of ATP and Mg^{2+} , which restored the mitochondria to their normal state (Rossi and Lehninger, 1964; Carafoli, Patriarca and Rossi, 1969). Thus ATP and Mg^{2+} were required to prevent Ca^{2+} and Pi loss.

Ca^{2+} accumulation was found to uncouple oxidative phosphorylation and such uncoupling continues only until nearly all the Ca^{2+} in the medium has been accumulated (Rossi and Lehninger, 1964).

When ATP was hydrolysed, Ca^{2+} and Pi were accumulated under limiting uptake conditions in the absence of substrate. (Bielawski and Lehninger, 1966). Ca^{2+} stimulated ATP hydrolysis proceeded at a high rate for a short period and then returned to a low resting rate. The Ca^{2+} and Pi accumulation coupled to ATP hydrolysis were shown to exhibit a similar stoichiometric relationship to electron transport supported accumulation (Bielawski and Lehninger, 1966), in contrast to the uncoupled ATP hydrolysis and Ca^{2+} accumulation under massive loading conditions.

Ca^{2+} accumulation in the presence of ATP, but in the absence of respiratory substrates, was inhibited by oligomycin but not by respiratory inhibitors or uncouplers of oxidative phosphorylation (Brierly, Murer and Green, 1963; Drahota et al, 1965; Lehninger, 1969; Tjioe, Bianchi and Haugaard, 1970). However both systems were inhibited by dicoumarol, while DNP only blocked the substrate supported accumulation. Respiratory inhibitors and uncoupling agents added to

mitochondrial medium after Ca^{2+} has been accumulated produced a rapid discharge of Ca^{2+} (Drahota et al, 1965; Carafoli et al, 1969).

Ca^{2+} was accumulated, without stimulating resting respiration, when the Ca^{2+} concentration in the medium was below $2\ \mu\text{M}$ (Carafoli, Rossi and Lehninger, 1965). Low efficiency of oxidative phosphorylation was achieved under resting state respiration (Carafoli et al, 1965). The Ca^{2+} : O ratio was very low, about 0.25.

Ca^{2+} efflux from mitochondria had the characteristics of an enzyme controlled process. It had a very high temperature coefficient (Drahota et al, 1965), was highly dependent on pH and salt concentration especially Na^+ and K^+ (Drahota and Lehninger, 1965; Rossi, Bielawski, Carafoli and Lehninger, 1966a), and uncoupling agents such as 2,4 DNP, dicoumarol and gramicidin greatly stimulated loss of Ca^{2+} from loaded mitochondria (Drahota et al, 1965; Chappell and Croft, 1965a; Croft and Chappell, 1965). The efflux of Ca^{2+} was accompanied by an uptake of H^+ from the medium in a ratio of 1:1 (Drahota et al, 1965; Chappell and Croft, 1965a and b; Croft and Chappell, 1965; Rossi, Bielawski, Carafoli and Lehninger, 1966a). Energy linked Ca^{2+} accumulation by mitochondria was accompanied by the movement of other ions so as to keep electrochemical balance in the intramitochondrial compartment. Other permeant anions such as acetate, propionate and arsenate may replace PO_4^{3-} and enter mitochondria passively with Ca^{2+} (Rasmussen, Chance and Ogata, 1965). Chloride, bromide and nitrate were found to be impermeants. Acetate uptake causes mitochondria swelling to the point of lysis, while in Ca^{2+} uptake, in the presence of Cl^- , there was no swelling (Chance, 1965; Rasmussen et al, 1965). Rasmussen

et al (1965) attributed the effect of swelling and shrinking to energy dependent movements of ions in and out of mitochondria.

Saris (1963) first showed that during respiratory stimulated Ca^{2+} uptake, the medium became more acid. This was suggested to be because H^+ was ejected on Ca^{2+} accumulation. This was confirmed by Engstrom and DeLuca (1964a), Chance (1965), Drahota et al (1965), Rasmussen et al (1965) and Rossi, Bielawski and Lehninger (1966b). Under normal conditions (pH 7.2, 80mM-NaCl) in the absence of PO_4^{3-} and other permeant anions, at least one proton (H^+) was ejected per Ca^{2+} ion accumulated (Drahota et al, 1965; Rossi et al, 1966b). 2,4 DNP and other uncoupling agents block the ejection of H^+ on Ca^{2+} addition to mitochondria, but oligomycin does not inhibit H^+ ejection or Ca^{2+} uptake. The $\text{H}^+:\text{Ca}^{2+}$ ratio varies considerably depending on condition (Gear and Lehninger, 1968). In the absence of permeant ions, the $\text{H}^+:\text{Ca}^{2+}$ ratio was highly dependent on Na^+ and K^+ content of medium. Increasing the pH of the medium also caused an increase, but a smaller one, in the $\text{H}^+:\text{Ca}^{2+}$ ratio in the absence of permeant anions (Gear and Lehninger, 1968). The $\text{H}^+:\text{Ca}^{2+}$ ratio decreased in the presence of permeant anions in the medium (Saris, 1963; Chance, 1965; Rasmussen et al, 1965; Rossi et al, 1966b). Thus during Ca^{2+} accumulation, at least two protons are ejected per pair electrons traversing each of the three energy conserving sites. Some observations suggest that the true maximum was four protons per site (Massari and Azzone, 1970). Accompanying H^+ ejection was alkalization of the mitochondria (Rossi et al, 1966b; Chance and Mala, 1966; Gear, Rossi, Reynafarje and Lehninger, 1967). This alkalization was shown to be

stoichiometric with H^+ ejected and with electron transport (Rossi et al, 1966b; Gear et al, 1967; Loyter, Christiansen, Steinsland, Saltzgaber and Racker, 1969). This only occurs in the presence of Ca^{2+} and respiration, and is discharged on the addition of 2,4 DNP (Rossi et al, 1966b; Gear et al, 1967).

During normal uptake of Ca^{2+} by mitochondria in the absence of valinomycin, no K^+ was ejected; however, when valinomycin was present the K^+ ejection followed an addition of Ca^{2+} (Carafoli, 1967; Ogata and Rasmussen, 1966; Drahota, Gazzotti, Carafoli and Rossi, 1969). If the medium already contained K^+ then little mitochondrial K^+ was ejected and the $H^+ : Ca^{2+}$ ratio was high (Gear and Lehninger, 1968). In an all Na^+ , valinomycin medium in the absence of phosphate, the sum of the K^+ ejected plus H^+ ejected nearly equalled the amount of Ca^{2+} accumulation (Rossi, Azzi and Azzone, 1967; Selwyn, Dawson and Dunnett, 1967).

When respiration dependent accumulation of Ca^{2+} in the presence of PO_4^{3-} occurred, then adenine nucleotides were also accumulated from the medium (Carafoli, Rossi and Lehninger, 1965). About one molecule of ATP or ADP was accumulated per 12 Ca ions. Atractyloside, an inhibitor of ATP accumulation by mitochondria, inhibited Ca^{2+} uptake in the presence of phosphate, but not in the absence of PO_4^{3-} (Carafoli, Rossi and Lehninger, 1965; Vignais, Duee, Colomb, Reboul, Cheruy, Barzu and Vignais, 1970; Vignais and Vignais, 1971). It has been shown that ATP is present in $Ca_3(PO_4)_2$ granules (Weinbach and Von Brand, 1965).

The pH and the concentrations of NaCl or KCl of the medium

have been shown to drastically modify the stoichiometric relationships between Ca^{2+} accumulation, oxygen uptake, and H^+ ejection in the absence of PO_4^{3-} or other permeant anions (Rossi and Azzone, 1965; Carafoli, Gamble and Lehninger, 1965a; Carafoli, Gamble, Rossi and Lehninger, 1966; Rossi et al, 1966a; Carafoli, Rossi, Gamble, Lehninger, 1967). The $\text{Ca}^{2+} : \sim$ accumulation ratio for respiratory jump in the absence of PO_4^{3-} was reported to be a function of concentration of NaCl or KCl in the medium, as well as pH. At pH 7.4 and 80mM-KCl or NaCl, with and without PO_4^{3-} , the $\text{Ca}^{2+} : \sim$ ratio was about 2. When salt concentration increases to 320mM at constant pH, with PO_4^{3-} absent, the $\text{Ca}^{2+} : \sim$ ratio increased to 5.0 or higher. While in the presence of PO_4^{3-} the ratio remains near 2.0 (Carafoli et al, 1965, 1967). If sucrose replaced salts, there was no increase in the ratio. When the pH was raised from 7.2 to 8.2., at constant NaCl of 80mM, there was a large increase in $\text{Ca}^{2+} : \sim$ ratio to over 10 (Carafoli et al, 1965, 1967). When PO_4^{3-} was present in the medium, then the ratio returned to normal value. There was no change in ADP:O ratios of oxidative phosphorylation when there were changes in pH and salt concentration (Carafoli et al, 1967). These findings may be related to the inhibition of Ca^{2+} efflux from loaded mitochondria by conditions of elevated pH and NaCl or KCl concentrations (Rossi et al, 1966a).

Rebound and incipient oscillations in the movement of Ca^{2+} in rat liver mitochondria at low salt concentrations were observed, in which Ca^{2+} rapidly accumulated and discharged and then reaccumulated, with the reverse process occurring for H^+ . (Carafoli, Gamble and Lehninger, 1965b). This occurs in the absence of any inflection of

resting oxygen uptake. This rebound did not occur in the presence of ATP, Mg^{2+} and sucrose and was blocked by rotenone (Carafoli, Gamble and Lehninger, 1966).

Substrates for Ca^{2+} accumulation have been shown not to accompany the process of Ca^{2+} uptake (Carafoli et al, 1965c).

Digitonin fragments of mitochondria have been shown to accumulate considerable quantities of Ca^{2+} , but the maximum amount was smaller than for intact mitochondria (Vasington, 1963). Furthermore the stoichiometry with oxygen uptake was nearly the same. There was no requirement for ADP or ATP. The uptake of Ca^{2+} is very rapid. However, sonic particles from rat liver mitochondria were found not to accumulate Ca^{2+} , nor eject or absorb H^+ on addition of Ca^{2+} (Gear, 1967). Beef heart particles prepared by sonication, on the other hand, did accumulate Ca^{2+} . Mitchell and Moyle (1965) explained this by proposing the particles had become everted vesicles.

Vasington and Greenawalt, (1964) showed that water-lysed rat liver mitochondria had lost their outer membrane and much of the mitochondrial matrix, but retained an unimpaired capacity for respiration linked accumulation of Ca^{2+} . These particles were unable to accumulate small amounts of Ca^{2+} in the absence of PO_4^{3-} . Maximal Ca^{2+} uptake by these ghosts required the presence of PO_4^{3-} , Mg^{2+} and ADP. Other nucleotide-5-diphosphates failed to replace ADP. It was concluded that this was due to the loss of the outer membrane or matrix component.

Particles of mitochondria prepared from beef heart, by swelling at $0^\circ C$ and thawing were shown to accumulate Ca^{2+} and PO_4^{3-} during respiration. These preparations required ADP or ATP in the system

(Penniston, Vande Zande and Green, 1966a and b).

Sr^{2+} has been shown to be accumulated by mitochondria in a process that is very similar to that involved in Ca^{2+} uptake under both massive and limited conditions (Chappell and Greville, 1963; Carafoli, Weiland and Lehninger, 1965; Carafoli, 1965a and b; Caplan and Carafoli, 1965). Both respiration-dependent and ATP-dependent Sr^{2+} uptake occur. The stoichiometry with oxygen uptake and ATP hydrolysis are similar to that for Ca^{2+} uptake (Carafoli, et al, 1965). Sr^{2+} and Ca^{2+} compete for uptake by rat liver mitochondria, Ca^{2+} being more avidly accumulated (Carafoli, 1965b). ATP dependent uptake of Sr^{2+} was more labile than Ca^{2+} ATP dependent uptake (Vasington, 1966). Under limited loading conditions, Sr^{2+} was less readily accumulated in the absence of PO_4^{3-} than Ca^{2+} (Carafoli, 1965b). In the presence of PO_4^{3-} , Sr^{2+} uptake was more complete and the stimulated respiration returned to resting state after Sr^{2+} uptake. However Sr^{2+} uptake did not require ATP for the maintenance in the presence of PO_4^{3-} , in contrast to Ca^{2+} uptake. Sr^{2+} inhibits mitochondrial swelling, promotes contraction and inhibits ATPase activity (Caplan and Carafoli, 1965).

Mn^{2+} uptake has not been extensively studied, but stimulates respiration only sluggishly (Carafoli, 1965a; Mela and Chance, 1968). The mechanism of Mn^{2+} uptake was similar to that of Ca^{2+} and Sr^{2+} . Mn^{2+} was not accumulated by intact mitochondria from most tissues, although beef heart mitochondria may be a special case (Brierly, Bachmann and Green, 1962).

Large electron dense osmophilic granules have been shown to

appear in the matrix of rat liver mitochondria when the mitochondria were massively loaded with Ca^{2+} and PO_4^{3-} (Lehninger et al, 1963; Greenawalt et al, 1964; Brierley and Slauterbach, 1964; Weinbach and Von Brand, 1965; Christiansen, Loyter, Steinsland, Saltzgaber and Racker, 1969). Such granules have been shown to be within mitochondria of rachitic and vitamin D-replete mucosal cells of rat intestine (Sampson et al, 1970) and in chondrocytes of bone (Martin and Matthews, 1969; Matthews et al, 1970). These granules were found to be deposited only when electron transport was available. They do not form in the absence of an energy source, no matter how high the Ca^{2+} concentration is in the medium. The granules were not formed when PO_4^{3-} was replaced by arsenate or acetate in the medium. Both a respiratory substrate and ADP or ATP are required for deposition. However Leblanc, Bourdain and Clauser (1970) reported that ADP was not associated with the precipitation of $\text{Ca}_3(\text{PO}_4)_2$. If ADP was omitted the mitochondria swelled and failed to accumulate more than a few hundred nmoles of Ca^{2+} /mg of protein. ADP or ATP cannot be replaced by other nucleotides. Since atractyloside inhibits ATP entry into mitochondria, it inhibited $\text{Ca}_3(\text{PO}_4)_2$ deposition. Adenine nucleotide concentration inside mitochondria has been shown to rise ten fold during accumulation of $\text{Ca}_3(\text{PO}_4)_2$ (Carafoli et al, 1965). The role of ADP or ATP in the formation of $\text{Ca}_3(\text{PO}_4)_2$ granules in the matrix was not clear, even though much research has been carried out (Rossi and Lehninger, 1964; Carafoli, Rossi and Lehninger, 1965; Caplan and Greenawalt, 1967; Lehninger, 1970). These granules have been shown in mitochondria in situ under normal and pathological conditions (Peachey,

1964; Reynolds, 1965). Three stages of energy linked deposition of Ca^{2+} in rat liver mitochondria have been found (Greenawalt et al, 1964; Greenawalt and Carafoli, 1966; Thomas and Greenawalt, 1968). At the first stage, very small granules appear on the inner surface of inner membrane (Brierley and Glauberbach, 1964). Secondly, granules appear in matrix, and thirdly, granules grow by accretion. Small $\text{Ca}_3(\text{PO}_4)_2$ granules do not appear from the dense granules that are already present in mitochondria and these dense granules do not have mineral deposits on microincineration of normal mitochondria (Rochetti-Pasquali, Greenawalt and Carafoli, 1969). These small $\text{Ca}_3(\text{PO}_4)_2$ granules were shown to be amorphous (Greenawalt et al, 1964; Thomas and Greenawalt, 1968). However when $\text{Sr}_3(\text{PO}_4)_2$ was deposited in mitochondria, it was crystalline (Greenawalt and Carafoli, 1966).

It has been postulated the process of calcification of hard tissues could be associated with mitochondria (Greenawalt et al, 1964; Halstead, 1969; Shapiro and Greenspan, 1969). This process may not only be associated with bone but may be widely distributed, e.g. egg-shells, in the formation of exoskeletons, hardening of plant tissues, and even in human in brain, blood vessels, kidney, and liver after injury. In mammalian bone, there has been recognized two phases of calcium phosphate - one as tricalcium phosphate which is amorphous, non-diffracting and more labile, and the second - crystalline hydroxyapatite (Posner, 1969). The tricalcium phosphate has been cited as an obligatory first stage to hydroxyapatite formation. The amorphous $\text{Ca}_3(\text{PO}_4)_2$ has been found to be more soluble than hydroxyapatite at pH 7.0. The concentration of Ca^{2+} and PO_4^{3-} in blood plasma are below the

solubility product for amorphous $\text{Ca}_3(\text{PO}_4)_2$. It has been also found that a substance formed in human tissues and excreted in urine can inhibit the formation of hydroxyapatite in vitro and in vivo (Barker, McPhillips, Lawrence, Doty, Pallante, Bills, Scott and Howard, 1970). This factor was also present in blood and milk and some tissues of normal individuals. This substance, may thus inhibit the formation of bone crystals.

Lehninger (1970) has proposed that the mitochondria were concerned in calcification as they concentrate Ca^{2+} and PO_4^{3-} in mitochondria from the cytoplasm, so as to exceed the solubility product of tricalcium phosphate. The $\text{Ca}_3(\text{PO}_4)_2$ insoluble amorphous granules in the mitochondria matrix may be precursors of the extracellular hydroxyapatite. But how do these granules pass to the extracellular sites where $\text{Ca}_3(\text{PO}_4)_2$ is deposited? It has been proposed that these granules were redissolved before deposition as hydroxyapatite in the extracellular compartment (Shapiro and Greenspan, 1969). Lehninger (1970) postulated that these granules left the mitochondria as aggregates and were stabilized by an inhibitor in the cytoplasm, and then passed through to the extracellular fluid to become attached to collagen fibrils. They later may be converted to hydroxyapatite, which possibly may necessitate the removal of the inhibitor.

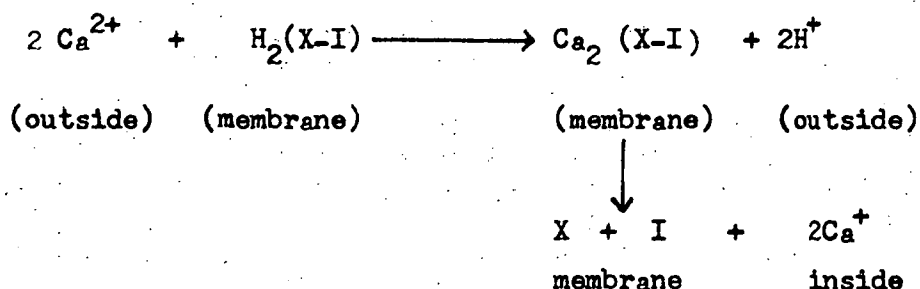
Studies on binding of Ca^{2+} to non-respiring rat liver mitochondria have revealed that there are at least two respiration independent Ca^{2+} binding sites (Reynafarje and Lehninger, 1969). One set has rather low affinity, but with numerous sites (40-60 nmoles/mg of mitochondrial protein), and a non-specific anionic binding to groups

of membrane proteins and lipids. The second type were less numerous (less than 1.0 nmole/mg of protein) and had a very high affinity for Ca^{2+} , which was comparable with the affinity that respiring mitochondria have for Ca^{2+} (Reynafarje and Lehninger, 1969). These high affinity binding sites bind Ca^{2+} , Sr^{2+} and Mn^{2+} in the absence of electron transport and ATP hydrolysis, but not Mg^{2+} , K^{+} or Na^{+} . These sites were only found in mitochondria of those species of plant or animal that were able to accumulate Ca^{2+} stoichiometrically during respiration, and were not present in blowfly muscle or yeast mitochondria (Reynafarje and Lehninger, 1969; Lehninger, Rossi, Carafoli and Reynafarje, 1969; Carafoli, 1970; Carafoli, Hansford, Sacktor and Lehninger, 1971). La^{3+} and Pr^{3+} were found to inhibit both respiration linked Ca^{2+} accumulation and high affinity Ca^{2+} binding (Mela and Chance, 1968, 1969; Mela, 1969; Lehninger, 1969; Selwyn et al, 1970; Carafoli, 1970). Ruthenium red was found to inhibit Ca^{2+} transport into rat liver mitochondria. It was suggested that, as this inhibitor was known to specifically react with mucopolysaccharides, it inhibited at the active centre of sites of mediation of mitochondrial Ca^{2+} transport (Moore, 1971).

Lehninger and Carafoli (1969) were able to extract from rat liver mitochondria, after osmotic shock with distilled water, a factor which showed high affinity Ca^{2+} binding as well as low affinity. This factor was able to bind Sr^{2+} , Mn^{2+} , and La^{3+} , and to a much less extent Mg^{2+} . The Ca^{2+} binding factor was heat labile and non-diffusible by dialysis (Lehninger, 1971). The factor was not affected by uncouplers or respiratory inhibitors, but was competitively inhibited for Ca^{2+}

binding by Sr^{2+} , Mn^{2+} , and La^{3+} .

Two basic models have been proposed for Ca^{2+} mediated transport across mitochondrial membranes. The first was proposed by Chance (1965) in which he postulated that Ca^{2+} combined directly and stoichiometrically with protonated, high energy intermediate, generated by electron transport so that there was a unidirectional transfer of Ca^{2+} across the mitochondrial membrane and a discharge of a high energy bond. The high energy intermediate released H^+ to the medium as Ca^{2+} was transport inward.



This model is linked as it is directional and there is an obligate stoichiometry between Ca^{2+} uptake and electron transport. In the second model proposed by Mitchell (1966), there was a passive reversible carrier, independent of the respiratory chain, that binds Ca^{2+} in a specific saturable manner and can transport in either direction in response to a transmembrane electrochemical gradient. This carrier is analogous to the carrier of ADP or ATP that is sensitive to atractyloside (Chappell, 1968). The carrier could be neutral or electrogenic. If neutral, it would carry a charge-compensating counteranion together with the Ca^{2+} or exchange Ca^{2+} from a compensating cation such as H^+ . In an electrogenic carrier, the electrical charge of Ca^{2+} would not be compensated and the carrier

would respond to either a chemical or electrical gradient. Such a carrier would not be a member of the respiratory train, not chemically coupled to the latter, and independent of electron transport in either direction. Thus both models depend on respiration for Ca^{2+} accumulation against a gradient, the former directly with chemical coupling, and the latter with a response to an electrochemical gradient generated by electron transport. Present evidence appears to support the latter model.

(1) The effect of vitamin D, parathyroid hormone and related compounds on calcium uptake and release by mitochondria

The mitochondria as previously outlined may be concerned in calcification of tissue. Vitamin D and PTH are known to effect the transport of Ca^{2+} and the calcification of bone. Thus these two compounds may also effect the uptake and release of Ca^{2+} from mitochondria.

Vitamin D, according to DeLuca, Gran, Steenbock (1957), DeLuca, Gran, Steenbock and Reiser (1957), DeLuca and Steenbock (1957) and DeLuca, Reiser, Steenbock and Kaesberg (1960) may effect the function and alter the structure of mitochondria.

The uptake of Ca^{2+} by rat liver and kidney mitochondria was not influenced by vitamin D (DeLuca and Engstrom, 1961; Engstrom and DeLuca, 1962; 1964a and b). However Finkelstein (1963) reported intestinal mitochondria from vitamin D-replete rats bound more Ca^{2+} at 0°C than vitamin D-deficient animals.

Vitamin D has a marked effect on the release of Ca^{2+} from liver and kidney mitochondria whether it was given in vivo (Engstrom and DeLuca, 1962; 1964a) or added in vitro, in unphysiological amounts

(2,000 I.U.) (DeLuca, Engstrom and Rasmussen, 1962). Related steroids of vitamin D added to the incubation medium did not have this effect. The release of Ca^{2+} may have been due to the unphysiological dose of vitamin D administered. The effect was not observed in heart mitochondria (DeLuca et al, 1962; Engstrom and DeLuca 1962; 1964a). The release was suggested to be due to an increased permeability of the mitochondria membrane by vitamin D.

Steroid hormones have been shown to affect the binding of Ca^{2+} by liver mitochondria (Kimberg and Goldstein, 1966), e.g. cortisol, deoxycorticosterone, aldosterone and 17β estradiol enhance Ca^{2+} binding, while testosterone and progesterone were ineffective.

The uptake of Ca^{2+} was not stimulated by PTH or vitamin D_3 (Engstrom and DeLuca, 1962; 1964a and b).

The release of Ca^{2+} from liver mitochondria was stimulated by the addition of PTH (final conc. 10^{-7}M) in vitro in the presence of vitamin D (40 I.U.) (Engstrom and DeLuca, 1962; DeLuca et al, 1962). A similar conclusion was also obtained by Rasmussen and Ogata (1966). This also concurs with Rasmussen's et al (1963) isolated intestinal villi experiments.

Rasmussen and Ogata (1966) found that PTH was required for maximal Mg^{2+} uptake. PTH stimulated respiration in the presence of Mg^{2+} and a permeant anion. This was also observed by Sallis, DeLuca and Rasmussen (1963) and Rasmussen, Sallis, Fang, DeLuca and Young (1964). The hormone also stimulates Pi uptake (Sallis, DeLuca and Rasmussen, 1963). The Mg^{2+} and Pi uptakes were found to be independent of vitamin D concentration. PTH was found to stimulate an ATPase

activity in mitochondria (DeLuca and Sallis 1965) and induce rapid and extensive mitochondrial swelling in medium deficient in Mg^{2+} (Utzumi, Sallis and DeLuca, 1966).

Peptides related to PTH and steroids related to vitamin D were shown to have no effect on Ca^{2+} uptake or release by mitochondria (DeLuca et al, 1962). However, Aurbach, Houston and Potts (1965) showed that PTH as well as polylysine, protamine and inert parathyroid peptides C_1 and C_2 stimulated Ca^{2+} release and ATPase activity.

Many of these studies on vitamin D and PTH effects have been carried out on mitochondria from tissue not associated with Ca^{2+} transport or calcification, although it is generally postulated that PTH and vitamin D serve to keep balance between the intracellular and extracellular Ca^{2+} concentrations by altering the cellular and subcellular distribution of ions (Halstead 1969).

CHAPTER 1ANIMALS, METHODS AND MATERIALS1.1. MANAGEMENT OF CHICKS

White crossbred (Leghorn and Australorp) cockerels were obtained on the day of hatching and were reared for 3 to 5 weeks in restricted artificial light, in an electrically heated tiered brooder, in an air conditioned room, on a diet deficient only in vitamin D (Sallis and Holdsworth, 1962a) with a slight modification to the diet in that $\text{Ca}_3(\text{PO}_4)_2$ was increased from 2 to 2.75% (w/w) and MnSO_4 from 0.02 to 0.05% (w/w). Unless stated otherwise the chicks were allowed free access to food and water at all times.

1.2. ASSESSMENT OF RICKETS IN CHICKS

Only those chicks were used which showed satisfactory signs of vitamin D-deficiency after 3 - 5 weeks, as judged from X-rays of the tibiotarsal articulation. If the tibia and small bones in tibiotarsal joint were more than a 2 mm apart, then the chick was classified as being rachitic.

The chicks which were used in all the experiments were within the weight range 100-180 g and of age 3 - 5 weeks.

1.3. SUPPLEMENTATION WITH VITAMIN D

Vitamin D_3 obtained from L. Light and Co. Ltd. (Colnbrook, England) was used to note its effect on rachitic chicks. The vitamin was administered as a single dose 800 I.U. in 200 μl propylene glycol by intramuscular injection from 16 to 72 h before the experiment.

1.4. CALCIUM ABSORPTION IN VIVO

1.4.1. Surgical Procedures

The duodenal loop preparation for the study of $^{45}\text{Ca}^{2+}/^{40}\text{Ca}^{2+}$ uptake was carried out under diethyl ether anaesthesia as described by Coates and Holdsworth (1961). The section of the duodenum used in these experiments was that from the pyloric sphincter to just before the bile duct, while the ileum was that section of the small intestine from the yolk sac to the upper end of the caecum.

1.4.2. ^{45}Ca Calcium loading of duodenal loop

In all the experiments carried out with this loop it was loaded by injecting 500 μl of $^{45}\text{Ca}^{2+}/^{40}\text{Ca}^{2+}$ solution containing varying concentrations of $^{40}\text{Ca}^{2+}$ from 0.08 mg to 4 mg/ml. The $^{45}\text{CaCl}_2$ obtained from Radiochemical Centre, Amersham, England was used by diluting with unlabelled CaCl_2 to give the required specific activity. The $^{45}\text{Ca}^{2+}/^{40}\text{Ca}^{2+}$ solution was prepared in 0.154M-NaCl at pH 6.5 with an approximate activity of 2 $\mu\text{Ci}/\text{mg}$. The radioactive solution was left in the loop for times from 2 to 60 min. At the end of this period of time, a blood or bone sample was taken for assessment of absorption. The blood sample being taken from the wing vein and the bone sample as the left tibia.

After removal from the bird, the loop was washed externally with 0.154M-NaCl to remove excess blood before the isolation of the mucosal cells. Then the loop was washed out after removal from the chick with the washing fluid of 0.25M-sucrose + 2mM- Mg^{2+} or 0.154M-NaCl at 4°C or room temperature. These two fluids were decided upon after a study of the best media for washing the gut out. The media

used for washing were 0.25M-sucrose, 0.25M-sucrose + 2mM-Mg²⁺, 0.154M-NaCl or Krebs-Ringer Phosphate + 0.25M-sucrose.

1.4.3 "Turnover" method of loading

A similar method to that described by Coates and Holdsworth (1961) for the perfusion of the duodenum except that in place of the cannuli modified polyethylene portex adaptors were placed through the incisions in the duodenum and then tied in place. The loop was reinserted into the cavity and the cavity was sewn up. The duodenum was washed out with warm (37°C) physiological saline and drained by blowing air through the loop. A tapered silicone rubber stopper was inserted in the distal adaptor. A 26 gauge needle, cut to the length of the stopper was placed through another silicone stopper, to which was attached 1 ml tuberculin syringe containing 500 µl ⁴⁵Ca²⁺/⁴⁰Ca²⁺ solution (0.4 mg/ml Ca²⁺, 2 µCi ⁴⁵Ca²⁺) in 0.154M-NaCl. The proximal stopper with needle attached was withdrawn, without allowing any flow back and was replaced by a normal silicone stopper. The chick was removed from the table and allowed to regain consciousness. The ⁴⁵Ca²⁺ solution was removed after 8 min by removing the stopper at each end and flushing out with 25 ml 0.154M-NaCl and removing the excess saline with a stream of air. 500 µl 0.154M-NaCl was then placed in the loop and left for a further 20 min. The loop was removed from the bird at the end of this period, washed out with 5 ml of 0.154M-NaCl. A bone sample was taken at the end of the 28 min period and treated as in 1.7.4.2.

1.4.4. The efflux of ^{45}Ca from plasma to intestinal lumen

200 μl , 20 μCi carrier free $^{45}\text{Ca}^{2+}$ (1 μg $^{40}\text{Ca}^{2+}$) was injected intraperitoneally into chicks. Ten and twenty minutes after the injection, blood samples were taken from the wing vein. At the end of the twenty minute period, the bird was anaesthetized and a portion of gut from the duodenum through to the ileum was washed out in situ with 20 ml 0.154M-NaCl. The washing fluid was collected by means of a tube inserted in the distal end of the ileum. The wash fluid was made up to 40 ml centrifuged and 1.0 ml of the supernatant was counted in 20 ml Bray's Phosphor (1.8.4). This portion of intestine was then removed, washed externally and then digested in 1 ml concentrated HNO_3 and when the solution turned clear, 500 μl concentrated HClO_4 was added with heating to dryness. The gut digest residue was made up to 10 ml 0.5M- HClO_4 and estimated for $^{45}\text{Ca}^{2+}$ and $^{40}\text{Ca}^{2+}$. A tibia was taken at 20 min for measurement of radioactive Ca^{2+} deposited in bone (1.7.4.1).

1.5. IN VITRO PREPARATION OF CELLS AND CELLULAR FRACTIONS

1.5.1. Isolation of mucosal cells

The duodenum was rapidly removed from the chick, washed externally with 0.154M-NaCl to remove excess blood and then internally with 0.25M-sucrose + 2mM- Mg^{2+} containing inhibitors when these were used. It was then placed on an ice-cold glass plate, slit longitudinally and scraped with the edge of a microscope slide to remove the mucosal cells from underlying muscle.

1.5.2. Preparation of homogenate

The mucosal cells from a duodenum were immersed immediately

in 7 ml ice-cold medium and homogenized with three strokes for 30 s at 1,200 rev/min in a Potter-Elvehjem homogenizer fitted with a teflon pestle. Various media were examined in an endeavour to obtain breakage of all the cells with minimum damage to the organelles. The media tried were (1) 0.25M-sucrose, (2) Krebs-Ringer Phosphate + 0.25M-sucrose, (3) Krebs-Ringer Phosphate, (4) 0.25M-sucrose + 2mM-Mg²⁺, (5) 0.25M-sucrose + 2mM-Mg²⁺ + 2×10^{-5} M-chloroquine, (6) Krebs-Ringer Phosphate + 6% (w/v) Dextran, (7) 54% (w/v) Urograffin + 5% (v/v) glycerol, (Williams and Ada, 1967), (8) 10% (w/v) Mannitol, (9) Organic solvents used with freeze-dried mucosal cells (Allfrey, Stern, Mirsky and Saetren, 1952). Only 0.25M-sucrose + 2mM-Mg²⁺ (with or without chloroquine) gave good separations and allowed estimations of Ca²⁺, PO₄³⁻ protein and enzymes to be made. When inhibitors were used then these were added to the 0.25M-sucrose + 2mM-Mg²⁺.

1.5.3. Cell fractionation

All procedures in this subsection were carried out at $2 \pm 1^\circ\text{C}$.

Materials were centrifuged in MSE 6L centrifuge (8 x 100) from 600 - 2,000g and Beckman ultracentrifuge L2-65D from 8,000 - 78,000g in 30 rotor and 79,000g - 269,000g in 65 rotor.

1.5.3.1. Crude Fractionation

1.5.3.1.1. Crude Nuclei and Main residue

The homogenate was centrifuged at 600g (r_{av} 18 cm) for 15 min. The supernatant was removed and the residue was rehomogenized in 5 ml of medium and recentrifuged at 1,000g (r_{av} 18 cm) for 15 min. The pellet was the main residue fraction.

For the isolation of crude nuclei, the homogenate was layered over 3 ml 0.34M-sucrose and centrifuged at 1,500g (r_{av} 18 cm) for 10 min. The supernatant was removed, and the residue resuspended in 3 ml 0.125M-sucrose + 2mM-Mg²⁺ homogenized gently and layered over 2.5 ml 0.34M-sucrose and centrifuged again at 1,500g (r_{av} 18 cm) for 10 min. The supernatants were combined for further fractionation to isolate mitochondria and microsomes.

The residue below the interface of 0.34M and 0.25M-sucrose was again resuspended in an equal volume of 0.125M-sucrose + 2mM-Mg²⁺ to make approximately 0.25M-sucrose. This suspension was layered over 0.44M-sucrose and centrifuged at 1,500g (r_{av} 18 cm) for 30 min. The supernatant above 0.44M/0.25M-sucrose interface was removed and the sedimented material was suspended in 0.125M-sucrose + 2mM-Mg²⁺ to give approximately 0.25M-sucrose, then layered over 1.0M-sucrose and centrifuged at 1,500g (r_{av} 18 cm) for 30 min. The material at the interface only was removed and retained as crude nuclei. A small amount of debris and some erythrocytes passed through the 1.0M-sucrose, but the crude nuclei collected at the interface.

The washings from above and below the nuclear fractions were collected separately and centrifuged at 13,000g (r_{av} 7.8 cm) for 10 min.

The supernatants and pellets were separately acidified and processed for ⁴⁰Ca²⁺ and ⁴⁵Ca²⁺ estimations.

1.5.3.1.2. Crude Mitochondria

The combined supernatants from the isolation of the main residue or crude nuclei were centrifuged at 14,000g (r_{av} 7.8 cm) for 10 min. The supernatant was again centrifuged at 14,000g for 10 min.

The combined sediments constituted the crude mitochondria. These pellets were either left at this stage for estimation of $^{45}\text{Ca}^{2+}$, $^{40}\text{Ca}^{2+}$ and protein or was further fractionated which is described in 1.5.3.2.2.

1.5.3.1.3. Crude microsomes

The supernatant from the mitochondria was centrifuged at 269,000g (r_{av} 5.7 cm) for 30 min to produce a pellet, a combination of heavy and light microsomes.

1.5.3.1.4. Final supernatant

This was supernatant after the removal of the microsomal fractions.

All the isolated fractions were resuspended in distilled water, acidified to 0.5M-HClO₄ with 5M-HClO₄. The fractions were investigated for $^{40}\text{Ca}^{2+}$, $^{45}\text{Ca}^{2+}$, Pi and protein (1.8).

1.5.3.2. Method used for further purification of:

1.5.3.2.1. Nuclei

Pure nuclei were obtained by density gradient method. The crude nuclei obtained above 1.0M/0.25M-sucrose interface were diluted with 0.125M-sucrose + 2mM-Mg²⁺ to 0.25M-sucrose, then layered over 0.58M-sucrose and centrifuged at 1,500g (r_{av} 18 cm) for 30 min. The sedimented material consisted of nuclei and had only a trace of cytochrome oxidase activity. In most experiments these nuclei were resuspended in 0.125M-sucrose and again layered over 1.0M-sucrose and centrifuged at 1,500g (r_{av} 18 cm) for 30 min. The interface contained nuclei free from cytochrome oxidase (fig.2).

FIGURE 2

Photographs of homogenate and pure nuclear fraction of chick duodenum mucosal cells

A A picture showing the microscopic appearance of the chick duodenum mucosal cell homogenate as seen by phase contrast. The homogenate was prepared as described in 1.5.1 and 1.5.2. 750 x

B A picture showing the microscopic appearance of chick duodenum mucosal cell nuclei as prepared from the cell homogenate as described in 1.5.1, 1.5.2, 1.5.3.1 and 1.5.3.2.1. 750 x

(The quality of the picture is due to the poor reproduction from the original photographs.)

FIGURE 2A

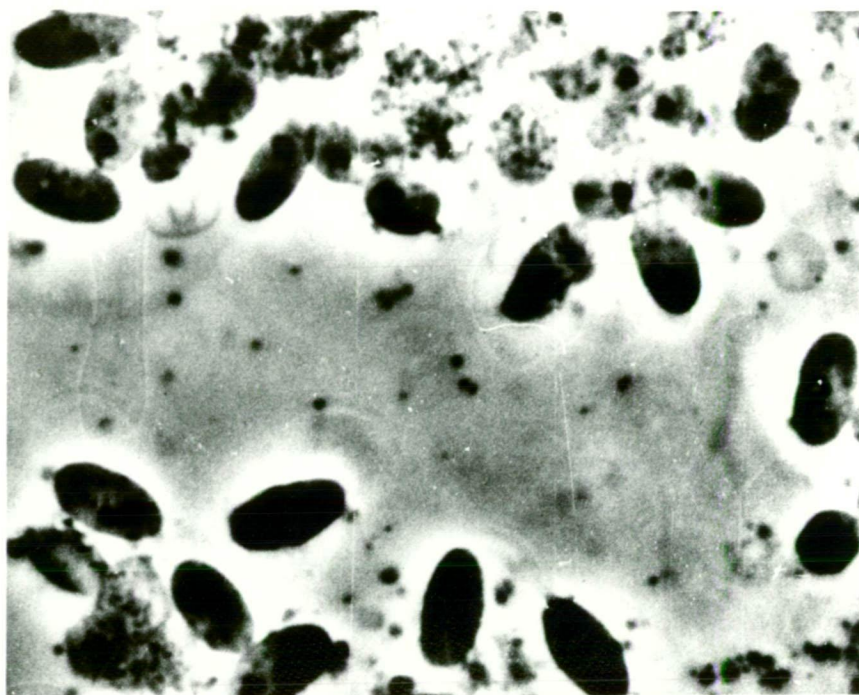
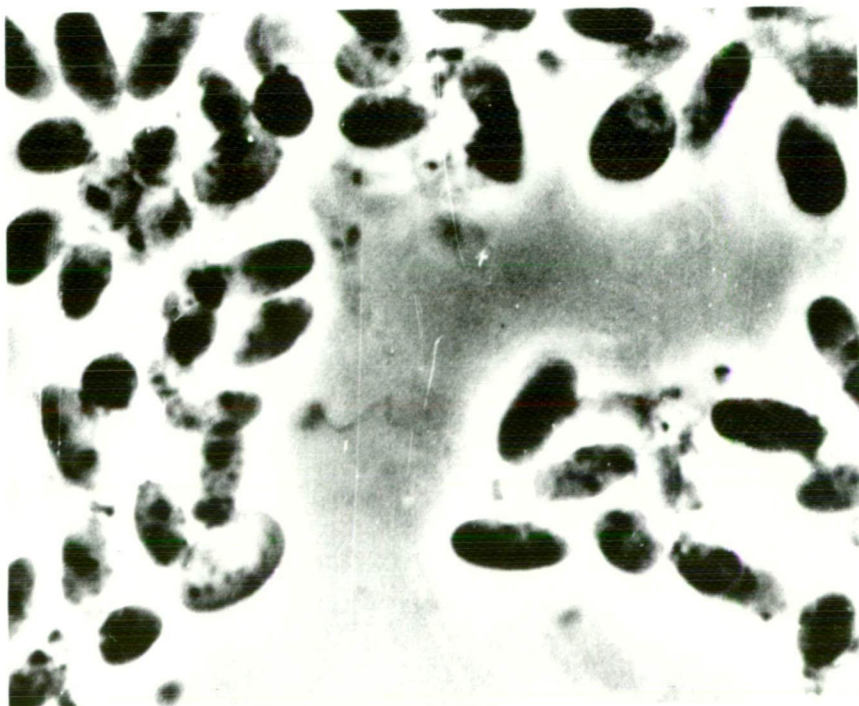


FIGURE 2B



1.5.3.2.2. Mitochondria

The crude mitochondria were resuspended in 0.25M-sucrose and layered over a linear gradient of 1.0 to 1.75M-sucrose. This gradient was produced by adding 50 ml of 1.75M-sucrose to a 50 ml conical flask. A rubber bung through which an 18 gauge and 20 gauge luer lock needles had been inserted was placed in the neck of the flask. A magnetic "flea" was added to the conical flask. To the 18 gauge needle was connected a 20 ml luer syringe without the plunger. A length of polyethylene tubing long enough to reach 10 cm below the base of the conical flask was added to the 20 gauge needle. The conical flask was placed on top of a magnetic stirrer and started mixing. To the 20 ml syringe was slowly added 25 ml 0.25M-sucrose. A syphon was started by applying reduced pressure to the end of the polyethylene tube, which was then placed against the inside wall of a cellulose nitrate tube immersed in ice, with the level of the outlet tube below the base of the flask. 25 ml of the gradient was collected ranging from 1.75 - 1.10M-sucrose. The efficiency of this linear gradient device was checked by using methylene blue solution as a test, by measuring the dilution of this substance by spectrometry.

The gradient was centrifuged in the swing out bucket rotor (SW 25.1) of a Beckmann L-2-65D centrifuge at 64,000g (r_{av} 9.1cm) for 2 h and brought to rest with the brake off. Three bands could be seen, the bulk of the mitochondria (referred to as heavy mitochondria) constituted a band at approximately 1.45M-sucrose. Above this was a "middle band" and at the junction of the 1.1M and 0.25M-sucrose there was a white fluffy layer referred to as the "top band". Any material lying at the

bottom of the tube or below the heavy mitochondria layer was insignificant and was discarded.

1.5.3.2.3. Heavy and Light Microsomes

The supernatant from the mitochondria separation was centrifuged at 33,500g (r_{av} 7.8 cm) for 20 min to yield the heavy microsomes. The supernatant was then centrifuged at 269,000g (r_{av} 5.7 cm) for 30 min to give the light microsomes and a final supernatant.

1.5.3.3. Isolation of mitochondria from either untreated or loaded duodena

When only mitochondria were required the homogenate was layered over 5 ml 0.34M-sucrose and centrifuged at 1,500g (r_{av} 18 cm) for 15 min. The layer above the 0.34M-sucrose was removed and centrifuged at 13,500g (r_{av} 7.8 cm) for 15 min. The sediment of mitochondria was resuspended in 0.25M-sucrose or 0.25M-sucrose + 5 mg/ml bovine serum albumin (Sigma Chemicals) and diluted as required for the particular experiment.

1.5.4. Preparation of calcium binding protein

1.5.4.1. From whole cells

The binding protein was isolated from rachitic chicken and chicks were given 800 I.U. vitamin D₃ in propylene glycol intramuscularly 48-72 h previously. The binding protein was prepared from duodena of these chicks by a method described by Wasserman and Taylor (1966) and Taylor and Wasserman (1967). The protein content of the extract was reduced by heating to 60°C for 10 min (Wasserman and Taylor, 1970). The denatured protein was removed by centrifugation at 11,000g (r_{av} 7.8 cm) for 10 min. To the supernatant was added

0.48g/ml $(\text{NH}_4)_2 \text{SO}_4$ slowly while stirring for 3 h. The suspension was centrifuged for 10 min at 11,000g (r_{av} 7.8 cm). The supernatant was dialysed with a number of changes of distilled water until no further precipitate was formed when 1M- BaCl_2 was added to dialysate. Then the protein was dialysed against 2 changes of 2 litres Wasserman's Tris buffer for 12 h. The binding protein solution was then concentrated to a volume equivalent to the number of chicks used to prepare the extract, by ultrafiltration using a Diaflo apparatus (Amicon Corporation, Massachusetts, U.S.A.) fitted with a membrane UM 10 which would allow the passage of molecules with molecular weight less than 10,000. A chelex test for Ca^{2+} binding by the protein was carried out upon these preparations (1.8.5). The amount of Ca^{2+} bound to the calcium binding protein was determined by means of atomic absorption spectrophotometry (1.8.2). The protein content of this preparation was measured by means of Biuret method (1.8.1). The $^{45}\text{Ca}^{2+}$ bound and $^{40}\text{Ca}^{2+}$ bound by the protein were calculated as nmoles/mg of protein.

1.5.4.2. Intracellular location of calcium binding protein

The amount and location of the calcium binding protein (CaBP) was compared in rachitic chicks and similar chicks given 800 I.U. of vitamin D_3 45 h previously. Mucosal cells were scraped from the duodena, homogenized in 0.25M-sucrose + 2mM- Mg^{2+} and the subcellular fractions were isolated as described in 1.5.3.1, and 1.5.3.2. The fractions that were sedimented were drained of sucrose and the pellets homogenized with Tris buffer (Wasserman and Taylor, 1966) and centrifuged at 11,000g (r_{av} 7.8 cm) for 30

min. The volume of the extracts was adjusted to give a final protein concentration 5-10 mg/ml. The final supernatant of the cell homogenate was dialysed against distilled water for 2 h to reduce the concentration of sucrose, heated at 60°C for 10 min and centrifuged at 10,000g (r_{av} 7.8 cm) for 20 min. All fractions were tested for Ca^{2+} binding *by* chelex 100 method (Wasserman and Taylor, 1966).

1.5.4.3. To isolate a mitochondrial calcium binding protein

Two groups of rachitic chicks were taken and one group was injected with 800 I.U. vitamin D_3 intramuscularly 68 h before the experiment. The duodena were removed from the chicks that had been starved overnight. The mucosal cells were scraped off and homogenized in 0.25M-sucrose. Crude mitochondria were prepared. The mitochondria pellet was resuspended in 0.25M-sucrose and again centrifuged at 13,500g (r_{av} 7.8 cm) for 10 min. The mitochondrial pellet was homogenized in Wasserman's Tris buffer, centrifuged at 13,500g (r_{av} 7.8 cm) for 10 min and the supernatant was made up to a volume equivalent to 1 ml/bird.

The supernatant remaining after the isolation of mitochondria from the original homogenate was centrifuged at 78,000g (r_{av} 7.8 cm) for 20 min to remove microsomes. The supernatant formed was heated to 60°C for 10 min and centrifuged at 11,000g (r_{av} 7.8 cm) for 30 min. This supernatant was treated with $(NH_4)_2SO_4$, centrifuged, dialysed and concentrated as described before (1.5.4.1). This supernatant was also tested for Ca^{2+} binding activity.

1.6. CALCIUM UPTAKE AND RELEASE BY ORGANELLES IN VITRO

1.6.1. Calcium uptake by homogenate of untreated mucosal cells

1.6.1.1. Inhibitor effects

An homogenate was prepared as described in 1.5.1 except the resuspended residue and the supernatant were combined and mixed.

Generally to 10 ml of the homogenate was added to 10 ml of media with σ^* without the inhibitor and stirred for 10 min at 0°C . After this preincubation 50-100 μl (1-20 μCi $^{45}\text{Ca}^{2+}/\text{ml}$, 2-800 μg Ca^{2+}/ml) was added while stirring for a further 5-20 min at 0°C . The homogenate was layered over 0.34M-sucrose with σ^* without the inhibitor and fractionated as described in 1.5.3 and 1.5.3.2.

1.6.2. Uptake of calcium by mitochondria

1.6.2.1. Centrifugation method

Mitochondria were prepared as described in section 1.5.3. They were adjusted to contain 10-20 mg protein/ml. 1 ml of mitochondrial suspension was added to 3 ml 0.25M-sucrose with σ^* without the inhibitor and stirred for 10 min at 0°C or 30°C , then 50 μl $^{45}\text{Ca}^{2+}/^{40}\text{Ca}^{2+}$ solution (4 μg $^{40}\text{Ca}^{2+}$, 0.2 μCi $^{45}\text{Ca}^{2+}$) was added and stirred for a further 10 min. The mitochondria were centrifuged down at 20,000g (r_{av} 7.8 cm) for 10 min at 0°C or 30°C . The $^{40}\text{Ca}^{2+}$, $^{45}\text{Ca}^{2+}$ and protein content of the residue and supernatant were determined.

1.6.2.2. Filtration method

Mitochondria were prepared as described in 1.5.3 and the suspension was adjusted to contain 3-6 mg/ml protein. 1 ml

mitochondrial suspension was mixed with 1 ml of 0.25M-sucrose generally buffered with 50mM-Tris Tricine pH 7.2 and containing 10 mg bovine serum albumin. Inhibitors or CaBP when they were used were incorporated in this 1 ml of sucrose solution. The mitochondria were preincubated in this medium for 5-10 min and then 25-50 μ l $^{45}\text{Ca}^{2+}$ ($4 \mu\text{g } ^{40}\text{Ca}^{2+}$, $0.2 \mu\text{Ci } ^{45}\text{Ca}^{2+}$) were added and stirred for 15 min at 0°C or 30°C . At intervals 0-15 min, 500 μ l samples were withdrawn and placed on 25 mm diameter, 0.65μ DAWP millipore filters in modified millipore membrane holders. The holder had had the upper tapered section removed to leave a ring. The millipore holder was positioned on top of a 7 cm 17 gauge luer lock hyperdermic needle, which had been inserted into a 1 cm rubber stopper, which in turn was placed in a 5 ml pyrex tube with side arm. The side arm was connected to a suction apparatus. By means of a manifold and taps, 12 such pieces of apparatus could be connected at one time and run in sequence. Five seconds before the 500 μ l sample was placed on the filter, the suction was turned on and left on for 90-120 s after the sample had been placed on the filter membrane. Immediately the suction was turned off, the membrane was removed and placed in a scintillation vial to which was added 5 ml Bray's Phosphor (1.8.5). 100 μ l of the filtrate from the side arm test tube was placed in a scintillation vial containing 5 ml Bray's Phosphor. The vials were then ready for counting. The mitochondria were retained on the membrane. The uptake of $^{45}\text{Ca}^{2+}$ added was calculated from $^{45}\text{Ca}^{2+}$ left in the supernatant.

1.6.3. Release of calcium by mitochondria

Loaded mitochondria were prepared as described in 1.5.3.3. These mitochondria were treated as in 1.6.2.1 or 1.6.2.2 except no $^{45}\text{Ca}^{2+}$ was added and there was no preincubation period. The % release was calculated from the amount of $^{45}\text{Ca}^{2+}$ present in the supernatant.

The effects of nucleotides, PTH, PGE, inhibitors and CaBP on the release of calcium by mitochondria were examined.

1.6.4. Transport by everted ileum

The distal third of the small intestine of the chick's ileum was everted and incubated as described by Sallis and Holdsworth (1962a). The everted sacs were incubated for 15 min at 0°C or 30°C in 5 ml Krebs-Henseleit bicarbonate buffer (Dawson and Elliott, 1959) containing 20mM glucose and 1mM $^{45}\text{CaCl}_2$ (5 μCi $^{45}\text{Ca}^{2+}$ /ml). The sac (serosal surface) contained 1 ml of the same solution but with $^{40}\text{Ca}^{2+}$. After 15 min the outside (mucosal surface) of the sac was washed by immersion in the buffer containing $^{40}\text{Ca}^{2+}$ and the sac reincubated with fresh serosal and mucosal fluids which were at the same concentration as above, but made with stable Ca^{2+} . After the second incubation at 30°C for 15 min, the $^{45}\text{Ca}^{2+}$ released to the serosal and mucosal fluids and remaining in the tissue was determined after Sallis and Holdsworth (1962a).

1.7. PREPARATION OF TISSUE FOR CHEMICAL ESTIMATIONS

1.7.1. Cell fractions

The cell fractions nuclei, mitochondria and microsomes were generally resuspended in distilled water and acidified to 0.5M

with 5M-HClO₄. However if enzyme assays were to be done on these fresh fractions, then they were resuspended in 0.25M-sucrose and a portion of the suspension was made acidic with 5M-HClO₄. After acidification the solutions were left overnight then centrifuged at 3,000 rev/min (r_{av} 8cm) in a Griffin Christ centrifuge for 10 min. The supernatant were removed and kept for $^{40}\text{Ca}^{2+}$, PO_4^{3-} and radioactivity measurements. Proteins precipitated from sucrose solutions were washed twice with 0.5M-HClO₄ to remove sucrose before dissolving in 1M-NaOH for protein estimation.

1.7.2. Final supernatant

The final supernatant was acidified to 0.5M-HClO₄ and centrifuged to remove the protein.

This supernatant was used direct for $^{40}\text{Ca}^{2+}$, $^{45}\text{Ca}^{2+}$ and Pi estimation (1.8). The precipitated protein was estimated by the biuret method (1.8).

1.7.3 Blood

Blood samples which had been taken at intervals from chicks from a wing vein using a heparinized 1 ml tuberculin syringe and placed in 1 ml heparinized test tubes, were centrifuged at 3,000 rev/min (r_{av} 8cm) in a Griffin Christ centrifuge for 10 min. The plasma was removed and the radioactivity measured by one of the following methods.

1.7.3.1. Method I

50 ul of the plasma was added to 10 ml of phosphor containing Triton X100 or X102 (Swift & Co) in toluene (Madson, 1969).

1.7.3.2. Method II

To 1 volume of plasma was added 4 volumes 0.5M-HClO₄ the

mixture centrifuged and 100 μ l of the supernatant added to 5 ml Bray's Phosphor (Bray, 1960).

1.7.4. Bones

The left tibia from a chick that had absorbed $^{45}\text{Ca}^{2+}$ was treated for measurement of $^{45}\text{Ca}^{2+}$.

1.7.4.1. Method I

The tibia was removed, cleansed of all adhering tissue, extracted respectively with ethanol, 50%/50% (v/v) ethanol and ether, and then ethyl ether, for 48 h then dried to a constant weight at 100°C. The bone was ashed at 800°C for 2 h, cooled in a desiccator and reweighed. The percentage ash was calculated as a check that the birds were rachitic eg 26-31% ash compared with normal birds 38-42%. The ash was powdered, and 25 mg was added to 500 μ l conc. HCl and heated to dryness. The residue was dissolved in 1 ml 0.5M- HClO_4 , 100 μ l of this solution was added to 5 ml Bray's Phosphor (1.8.5). The radioactivity was expressed as counts/min/ashed tibia.

1.7.4.2. Method II

The tibia was removed, cleansed of tissue, dehydrated and defatted in ethanol/ether mixture 50%/50% (v/v) for 1 day. It was broken into 2 pieces, placed in 20 ml P.O.P. toluene (Dallwitz and Norman, 1968) and allowed to soak in the scintillator for at least 4 h before counting. The results were expressed as counts/min/whole tibia. This method was only used as a check that vitamin D_3 had increased calcium absorption. There was usually a 3 to 5 fold increase in C.P.M. when the rachitic tibia was compared with

vitamin D₃-treated tibia taken at 20 min after loading.

1.7.5. Gut digestion

Either the muscle + cells or mucosal cells were digested in 1.0-3.0 ml concentrated nitric acid by heating till clear and then 0.5-2.0 ml concentrated perchloric acid was added. This was further heated until dry. The residue was dissolved in 1 ml 0.5M-perchloric acid and 100 μ l of this solution was counted and 500 μ l used for Ca²⁺ estimation.

1.8. CHEMICAL ESTIMATIONS

1.8.1. Protein by biuret method

Protein was estimated by the biuret method of Gornall, Bardawill and David (1949). Protein fractions precipitated by acid were redissolved in 1M-NaOH for this estimation. For estimation of mitochondrial protein in suspension, 100 μ l was used in 2 ml Biuret + 900 μ l distilled water. Crystalline bovine serum albumin was used as the standard and a separate calibration curve was prepared with sucrose if the protein fractions contained sucrose.

1.8.2. Calcium

A volume of supernatant from the acidified fractions was added to La³⁺ acetate pH 4.7 to give a final concentration of La³⁺ 10,000 ppm (Willis, 1961). The resultant solution was thoroughly mixed, centrifuged to remove any precipitate and then the Ca²⁺ concentration determined by using Techtron AAL00 atomic absorption spectrophotometer at 422.7 nm. A standard solution of CaCl₂ was prepared from calcium carbonate (B.D.H.-A.R. Grade) by heating for 8 h at 500°C, weighed and dissolved in a calculated amount of dilute

HCl to give 40 mg Ca^{2+} /ml (w/v). This was used as a reference standard for non-radioactive Ca^{2+} estimations diluted to the appropriate concentration and containing 0.5M- HClO_4 and La^{3+} 10,000 ppm.

1.8.3. Inorganic phosphate

Inorganic phosphate was estimated in acid deproteinized solutions by the method of Fiske and Subbarow as modified by Taussky and Shorr (1953).

1.8.4. Measurement of radioactivity

Radioactive samples were counted in Bray's Phosphor or POP Toluene or Triton Toluene. Generally, a sample of the acidified supernatants of fractions or the mitochondrial supernatant were counted in 5 ml Bray's Phosphor (Bray, 1960). In the case of the mitochondrial experiments the millipore membranes, the filtrates samples were counted in 5 ml Bray's Phosphor without further treatment. Beckmann model LS200 liquid scintillation counter fitted with an external standard was used for radioactivity measurement. Counts obtained were corrected for quenching.

Radioactive Ca^{2+} was used as a tracer for Ca^{2+} exchanges and Ca^{2+} transfer across biological membrane and for testing the binding activity of CaBP and brush borders. The specific activity of the $^{45}\text{Ca}^{2+}$ solutions prepared decreased with time and no attempt was made to use the same amount of radioactivity in each experiment. Therefore, the radioactivities which were taken to reflect Ca^{2+} exchanges, are only consistent within each experiment and cannot be compared directly between experiments although ratios to control

level may be generally compared.

1.8.5 Calcium binding measurements by the chelex method

The chelex test for Ca^{2+} binding proteins was performed on microsomal cell extracts and mitochondrial extracts. In this test the chelex and the proteins are in competition for $^{45}\text{Ca}^{2+}$ placed in the medium. The method used was modified from Wasserman and Taylor (1966) and Taylor and Wasserman (1967), in that excess $^{45}\text{Ca}^{2+}$ solution was added to and mixed with CaBP and protein, before adding chelex. The chelex had previously been washed several times with 0.1M-Tris-HCl pH 7.4 and finally suspended in Wasserman's Tris Buffer at pH 7.4 (Wasserman and Taylor, 1966). After removal of the 'free' Ca^{2+} by absorption on to chelex 100, the amount of $^{45}\text{Ca}^{2+}$ bound to protein was determined by liquid scintillation counting of the supernatant.

1.8.6 Deoxyribonucleic acid assay

100 μl to 1.0 ml sample were treated with 4 ml 10% (w/v) trichloroacetic acid and centrifuged. The supernatant was removed and the residue treated with 1 ml 0.5M-perchloric acid and 2 ml diphenylamine reagent (Burton, 1956). This mixture was incubated at 37°C for 16 h. The absorbance was read at 600 nm.

1.9 ENZYME ESTIMATIONS

The estimations of enzymatic activity were done in freshly prepared organelles suspended in 0.25M-sucrose.

1.9.1 Cytochrome Oxidase (E.C. 1.9.3.1)

0.50 mg/ml cytochrome C in 50mM-phosphate buffer pH 7.5 was reduced with 50 mg palladised asbestos and hydrogen gas. To

3 ml of the reduced cytochrome C was added between 25-100 μ l of the cellular fraction. The rate of oxidation by each fraction of the cell homogenate was studied at 549 nm and at 30°C for 1 min (Smith, 1955). The rate of reoxidation was linear for at least 30 s. This rate was used to calculate the μ moles cytochrome C oxidised/min/mg protein.

1.9.2. Alkaline phosphatase (E.C. 3.1.3.1)

Between 25-100 μ l of the fractions from the homogenate were incubated with 3 ml 10mM-p-nitrophenyl phosphate in 50mM-glycine buffer containing 2mM-Mg²⁺ pH 9.2 at 30°C. The release of p-nitrophenyl was followed continuously by measuring the optical density at 405 nm (Morton, 1955). The number of μ moles p-nitrophenyl (or phosphate) released per min/mg protein were calculated.

1.9.3. Acid phosphatase (E.C. 3.1.3.2)

50-100 μ l samples of each fraction or tube were incubated for 5 min at 30°C with 3 ml 5-10mM-p-nitrophenyl phosphate in 0.1M-sodium citrate buffer with and without 50mM alloxan pH 4.8 for 3-30 min. The reaction was stopped by the addition of 4 ml 0.10-0.50M-NaOH and the p-nitrophenyl released measured at 405 nm. The amount or number of μ moles of p-nitrophenyl released per min/mg protein were calculated (Schmidt, 1955).

1.9.4. 5' Nucleotidase (E.C. 3.1.3.5)

25-100 μ l samples from the fractions were incubated with 3 ml 5mM-AMP in 0.1M-glycine buffer at pH 8.5+10mM-Mg²⁺ at 30°C for 10 min (Heppel and Halmore, 1955). The amount of inorganic phosphate released was measured as described in 1.8.3. The number of μ moles

Pi released per min/mg protein were calculated.

1.9.5. Malic dehydrogenase (E.C. 1.1.1.37)

10-100 μ l of sample were incubated for 3 minutes at 37°C with 2.8 ml reaction medium containing 20mM-Tris-HCl pH 8.5, 10mM-EGTA-Tris pH 7.1, 30mM-nicotinamide, 0.1% Triton-X100, 50mM-sodium malate, 0.7mM-NAD and 5mM-KCN, pH 8.5. The reaction was measured at 340 nm against reaction medium without the substrate. The results are expressed as change in absorbance per min per mg protein. This method was modified from that reported by Beaufay, Bendall, Baudhuin and De Duve (1959).

1.9.6. Succinic dehydrogenase (E.C. 1.3.99.1)

100 μ l-1.0 ml sample were incubated with 250 μ l 0.2mM-sodium succinate (pH 7.4) + 250 μ l 0.2M-sodium phosphate buffer pH 7.4 + 100 μ l 1% (w/v) 3(4,5 dimethyl thiazolyl-2-)-2,5 diphenyl tetrazolium bromide and 200 μ l distilled water for 15 min at 37°C. To stop the reaction 1 ml 10% (w/v) trichloroacetic acid were added. To develop the colour 4 ml tetrahydrofuran was added. The absorbance was measured at 410 nm.

1.10. Statistical treatment of results

Significance was attributed to differences at $P = 0.05$ level using the "t" test as described by Bradford Hill (1961).

Significance is usually only remarked where this might appear doubtful for the conclusions drawn, since in many cases it is obvious from the differences alone.

CHAPTER 2

THE EFFECT OF VITAMIN D₃ ON CALCIUM DISTRIBUTION IN THE DUODENUMIntroduction

Prior to examining the mechanism of calcium transport in the duodenum of chicks it was first necessary to establish methods which would show the true distribution of the subcellular Ca²⁺ in vivo and in which fraction the turnover occurred.

Part 1 reports the effect of various washing media on the distribution of Ca²⁺ in the duodenum from rachitic and vitamin D-replete chicks. This was used as a guide in later experiments on the subcellular distribution of Ca²⁺.

In part 2.1 and 2.2 the subcellular distribution of Ca²⁺ within the duodenum was studied after Ca²⁺ absorption had taken place, for various time intervals, and at different concentrations of Ca²⁺. Part 2.3 examines the effect of vitamin D₃ on the turnover of ⁴⁵Ca²⁺ in the mucosal cells.

There was an attempt to purify the organelles obtained and to look at the Ca²⁺ distribution in these organelles in part 3. The enzyme purity of each fraction was tested and related to Ca²⁺ distribution. The effect of vitamin D₃ on the distribution of Ca²⁺ in these subcellular fractions and also its effect on enzyme activities was examined.

PART 12.1 The effect of various washing media on the distribution of calcium in the mucosal cells of the duodenumMETHOD

Calcium absorption by rachitic and vitamin D-replete chick duodenal mucosal cells was studied by method previously described in 1.4.1, 1.4.2, 1.5.1 and 1.5.2. The Ca^{2+} content in the mucosa of the duodenum from rachitic and vitamin D_3 -replete chick was examined and compared.

RESULTS

After leaving 2 mg $^{45}\text{Ca}^{2+}$ in the duodenal loops of rachitic and vitamin D_3 -replete chicks for 20 min, then the loops were washed out in situ with one of the following ice-cold solutions (i) 0.25M-sucrose, (ii) 0.25M-sucrose containing 2mM- Mg^{2+} , (iii) Krebs-Ringer phosphate solution containing 0.25M-sucrose and (iv) 0.154M- NaCl . The loops were then quickly removed, mucosal cells scraped off and the $^{40}\text{Ca}^{2+}$, $^{45}\text{Ca}^{2+}$ and protein contents were determined (Table 1). Table 1 shows that although the vitamin D-replete chicks were absorbing 3 times as much $^{45}\text{Ca}^{2+}$ as the rachitic chicks, as shown by $^{45}\text{Ca}^{2+}$ in the tibia, the amount of $^{45}\text{Ca}^{2+}$ accumulated by the mucosal cells was the same in both groups. Similar results were obtained when the loop was chilled with ice in situ before washing with ice cold solution, or even if the loops were washed out with solutions at room temperature.

TABLE 1

Calcium in mucosa of duodenum during absorption in vivo

500 μ l $^{45}\text{Ca}^{2+}/^{40}\text{Ca}^{2+}$ solution (2 mg, 5 μ Ci) were placed in the duodenal loop of starved chicks for 20 min (1.4.1 and 1.4.2). The duodenal loops were washed out in situ with 5 ml of these ice-cold solutions. The mucosal cells were isolated (1.5.1) and homogenized in the appropriate medium (1.5.2). The homogenate was assayed for $^{45}\text{Ca}^{2+}$, $^{40}\text{Ca}^{2+}$ and protein (1.7.1 and 1.8). The bone samples were treated as described in 1.7.4.1. These results are the average of duplicate duodena.

Wash solution	Type of Chick	Protein mg	$^{40}\text{Ca}^{2+}$ μ g	C.P.M.	C.P.M./ashed tibia
0.25M-sucrose	Vitamin D ₃ -treated Rachitic	222	423	639,000	10,400
		250	429	633,000	3,200
0.25M-sucrose + 2mM-Mg ²⁺	Vitamin D ₃ -treated Rachitic	304	625	917,000	13,400
		327	850	1,349,000	2,600
Krebs-Ringer-Phosphate + 0.25M-sucrose	Vitamin D ₃ -treated Rachitic	215	340	500,000	13,200
		224	407	555,000	3,300
0.154M-NaCl	Vitamin D ₃ -treated Rachitic	242	514	765,000	14,200
		295	519	780,000	2,190

PART 2

The effect of time, concentration of calcium and 'turnover' of calcium on the distribution of calcium in the mucosal cell

Part 2.1

2.2.1 The effect of various time intervals of loading on the subcellular distribution of calcium within the duodenum

METHODS

The duodenal loops were loaded for 2, 5, 8, 20 and 60 mins. These loops were treated as described in 1.4.1 and 1.4.3. The homogenate was prepared as described in 1.5.1 and 1.5.2 and fractionated as described in 1.5.3.1. The final supernatants were treated as described in 1.7.2. A bone sample was taken from chicks and treated as described in 1.7.4.2.

RESULTS

The endogenous Ca^{2+} presence in the subcellular fraction is shown in Table 2. The amount of endogenous Ca^{2+} present in mucosal cells of chicks starved overnight was similar for both rachitic and vitamin D_3 -replete intestine.

During absorption of $^{45}\text{Ca}^{2+}$ there was no detectable difference in the accumulation of $^{45}\text{Ca}^{2+}$ in bone until after 8 min and this accumulation was most marked at 20 min and 60 min (Table 3, 4, 5, 6 and 7).

At 20 min and 60 min, when 3 to 5 fold more $^{45}\text{Ca}^{2+}$ was being absorbed by vitamin D_3 -replete chicks, there was in fact more $^{45}\text{Ca}^{2+}$ accumulating in the mitochondria and nuclei of rachitic chicks. Before 20 min and in some experiments at 20 min (e.g. in Table 1) no differences were found in the amount of $^{45}\text{Ca}^{2+}$ accumulated by various

organelles. The soluble Ca^{2+} in the mucosal cells does not appear to alter from the endogenous level as compared to that present during absorption (Table 2 - 7), however the $^{45}\text{Ca}^{2+}$ content of supernatant from the vitamin D-replete chick contains more than the rachitic fraction ($D_3 : R = 2.4 : 1.0$) as an average of 23 experiments.

There appears to be about $20 \mu\text{g } \text{Ca}^{2+}$ in the final supernatant from the mucosal cells of a duodenum. Assuming these cells contain 1 ml of water, the concentration of Ca^{2+} in the soluble phase would be about $0.5 \times 10^{-3} \text{ M}$.

TABLE 2

The subcellular distribution of calcium in the mucosal cell of the duodenum (not loaded with calcium)

The loop from starved chicks was washed externally and internally after removal from the chick. The mucosal cells were isolated, homogenized in 0.25M-sucrose + 2mM-Mg²⁺ (1.5.1 and 1.5.2) and fractionated (1.5.3.1).

The results expressed as per duodenum are the average for 6 duodena.

Fraction	Type of Chick	Protein mg	⁴⁰ Ca ²⁺ μg
Crude Nuclei	Vitamin D ₃ -treated	87	68
	Rachitic	75	72
Crude Mitochondria	Vitamin D ₃ -treated	40	45
	Rachitic	38	48
Microsomes	Vitamin D ₃ -treated	36	15
	Rachitic	31	14
Final Supernatant	Vitamin D ₃ -treated	71	20
	Rachitic	62	21

TABLE 3

The subcellular distribution of calcium in mucosa of duodenum during absorption in vivo for 2 minutes

500 μ l $^{45}\text{Ca}^{2+}/^{40}\text{Ca}^{2+}$ (2 mg, 5 μ Ci) were placed in the duodenal loop of starved chicks for 2 min (1.4.1 and 1.4.2). The gut was removed, washed internally and externally and mucosal cells isolated, homogenized in 0.25M-sucrose + 2mM-Mg $^{2+}$ and fractionated (1.5.1, 1.5.2 and 1.5.3.1). A bone sample was taken from each chick and treated as described in 1.7.4.2. The results expressed as per duodenum are the average for 3 duodena.

Fraction	Type of Chick	Protein mg	$^{40}\text{Ca}^{2+}$ μ g	C.P.M.	C.P.M./ $^{40}\text{Ca}^{2+}$ μ g
Crude Nuclei	Vitamin D $_3$ -treated	97	127	358,370	2,827
	Rachitic	97	135	410,172	3,075
Crude Mitochondria	Vitamin D $_3$ -treated	51	65	181,363	2,706
	Rachitic	39	54	133,635	2,370
Microsomes	Vitamin D $_3$ -treated	22	9	10,826	1,196
	Rachitic	20	7	6,161	841
Final Supernatant	Vitamin D $_3$ -treated	62	10	5,947	638
	Rachitic	52	10	3,559	420

$^{45}\text{Ca}^{2+}$ accumulated by the tibia were vitamin D $_3$ chick 471 C.P.M./whole tibia and rachitic chick 478 C.P.M./whole tibia.

TABLE 4

The subcellular distribution of calcium in mucosal cells of the duodenum during absorption in vivo for 5 minutes

500 μ l $^{45}\text{Ca}^{2+}/^{40}\text{Ca}^{2+}$ solution (2 mg, 5 μ Ci) were placed in the duodenal loop of starved chicks for 5 min (1.4.1 and 1.4.2). After removal from the chick, the loop was washed externally and internally and the mucosal cells were then isolated, homogenized in 0.25M-sucrose + 2mM- Mg^{2+} and fractionated (1.5.1, 1.5.2 and 1.5.3.1). A bone sample was taken from each chick and treated as described in 1.7.4.2. The results expressed as per duodenum are the average for 3 duodena.

Fraction	Type of Chick	Protein mg	$^{40}\text{Ca}^{2+}$ μ g	C.P.M.	C.P.M./ $^{40}\text{Ca}^{2+}$ μ g
Crude Nuclei	Vitamin D ₃ -treated	78	176	228,754	1,324
	Rachitic	81	159	205,518	1,294
Crude Mitochondria	Vitamin D ₃ -treated	31	69	113,080	1,598
	Rachitic	24	44	47,695	1,103
Microsomes	Vitamin D ₃ -treated	32	12	11,019	886
	Rachitic	25	10	5,176	499
Final Supernatant	Vitamin D ₃ -treated	49	10	2,133	213
	Rachitic	47	8	313	39

$^{45}\text{Ca}^{2+}$ accumulated by the tibia were vitamin D₃ chick 1,303 C.P.M./whole tibia and rachitic chick 820 C.P.M./whole tibia.

TABLE 5

The subcellular distribution of calcium in mucosal cells of the duodenum during absorption in vivo for 8 minutes

500 μ l $^{45}\text{Ca}^{2+}/^{40}\text{Ca}^{2+}$ solution (2 mg, 5 μ Ci) were placed in the duodenal loop of starved chicks for 8 min. After removal of the loop, it was washed internally and externally and the mucosal cells were isolated, homogenized in 0.25M-sucrose + 2mM- Mg^{2+} and fractionated (1.5.1, 1.5.2 and 1.5.3.1). A bone sample was removed from each chick and treated as described in 1.7.4.2. The results expressed as per duodenum are the average of 6 duodena.

Fraction	Type of Chick	Protein mg	$^{40}\text{Ca}^{2+}$ μ g	C.P.M.	C.P.M./ $^{40}\text{Ca}^{2+}$ μ g
Crude Nuclei	Vitamin D ₃ -treated	66	63	158,799	2,532
	Rachitic	74	86	175,279	2,091
Crude Mitochondria	Vitamin D ₃ -treated	41	49	155,340	3,189
	Rachitic	35	36	91,970	2,614
Microsomes	Vitamin D ₃ -treated	33	14	22,062	1,570
	Rachitic	31	11	12,394	1,143
Final Supernatant	Vitamin D ₃ -treated	59	26	12,886	491
	Rachitic	62	19	8,089	434

$^{45}\text{Ca}^{2+}$ accumulated by the tibia were vitamin D₃ chick 7,079 C.P.M./whole tibia and rachitic chick 5,296 C.P.M./whole tibia.

TABLE 6

The subcellular distribution of calcium in the mucosal cells of the duodenum during absorption in vivo for 20 minutes

500 μ l $^{45}\text{Ca}^{2+}/^{40}\text{Ca}^{2+}$ solution (2 mg, 5 μCi) were placed in the duodenal loop of starved chicks for 20 min. After removal of the loop, it was washed internally and externally and the mucosal cells were isolated, homogenized in 0.25M-sucrose + 2mM- Mg^{2+} and fractionated (1.5.1, 1.5.2 and 1.5.3.1). A bone sample was removed from each chick and was treated as described in 1.7.4.2. The results expressed as per duodenum are the average for 2 duodena.

Fraction	Type of Chick	Protein mg	$^{40}\text{Ca}^{2+}$ μg	C.P.M.	C.P.M./ $^{40}\text{Ca}^{2+}$ μg
Crude Nuclei	Vitamin D_3 -treated	40	64	19,950	311
	Rachitic	40	72	28,198	391
Crude Mitochondria	Vitamin D_3 -treated	65	208	134,072	644
	Rachitic	61	216	156,190	723
Microsomes	Vitamin D_3 -treated	32	24	3,164	131
	Rachitic	37	25	3,514	140
Final Supernatant	Vitamin D_3 -treated	54	18	3,597	199
	Rachitic	62	12	1,841	153

$^{45}\text{Ca}^{2+}$ accumulated by the tibia were vitamin D_3 chick 10,576 C.P.M./whole tibia and rachitic chick 2,075 C.P.M./whole tibia.

TABLE 7

The subcellular distribution of calcium in the mucosal cell of the duodenum during absorption in vivo for 60 minutes

500 μ l $^{45}\text{Ca}^{2+}/^{40}\text{Ca}^{2+}$ solution (2 mg, 5 μ Ci) were placed in the duodenal loop of starved chicks for 60 min. After removal of the loop, it was washed internally and externally and the mucosal cells were isolated, homogenized in 0.25M-sucrose + 2mM-Mg $^{2+}$ and fractionated (1.5.1, 1.5.2 and 1.5.3.1). A bone sample was removed from each chick and treated as described in 1.7.4.2. The results expressed as per duodenum are the average for 3 duodena.

Fraction	Type of Chick	Protein mg	$^{40}\text{Ca}^{2+}$ μ g	C.P.M.	C.P.M./ $^{40}\text{Ca}^{2+}$ μ g
Crude Nuclei	Vitamin D $_3$ - treated	17	45	107,461	2,388
	Rachitic	17	73	260,263	3,565
Crude Mitochondria	Vitamin D $_3$ - treated	45	82	196,564	2,397
	Rachitic	46	125	491,909	3,935
Microsomes	Vitamin D $_3$ - treated	38	15	14,689	979
	Rachitic	39	13	20,838	1,602
Final Supernatant	Vitamin D $_3$ - treated	49	15	10,121	674
	Rachitic	53	15	10,187	699

$^{45}\text{Ca}^{2+}$ accumulated by the tibia were vitamin D $_3$ chick 42,358 C.P.M./whole tibia and rachitic chick 14,429 C.P.M./whole tibia.

PART 2.22.2.2. The effect of varying the calcium concentration in the lumen on the subcellular distribution of calcium

In Parts 1 and 2.1 with 2 mg $^{40}\text{Ca}^{2+}$ placed in the duodenal loops there was no significant difference in the amount of $^{45}\text{Ca}^{2+}$ accumulated between rachitic and vitamin D_3 -replete chicks intestinal cells. In this section, an attempt was made to show up differences in accumulation, by using lower concentrations of $^{40}\text{Ca}^{2+}$ and by studying the absorption over a short time period.

METHOD

The mucosal cells of duodenal loops of chicks were loaded with 500 μl $^{45}\text{Ca}^{2+}/^{40}\text{Ca}^{2+}$ solution (2 μCi) containing 0.5 mg or 5 mg $^{40}\text{Ca}^{2+}$ for 5 min and 2 mg or 0.04 mg $^{40}\text{Ca}^{2+}$ for 2 min. The cells were isolated (1.5.1), homogenized (1.5.2) and then treated for $^{40}\text{Ca}^{2+}$, $^{45}\text{Ca}^{2+}$ and protein estimation (1.7.1 and 1.8).

RESULTS

As the concentration of $^{40}\text{Ca}^{2+}$ presented for absorption increased, the amount of $^{45}\text{Ca}^{2+}$ accumulated by the intestinal cells increased as shown by the $^{40}\text{Ca}^{2+}$ /protein ratio in Table 8. At times of 5 min or less, the amount of $^{45}\text{Ca}^{2+}$ found in bones did not always show rachitic to vitamin D_3 -replete difference. Again as found in part 1 and 2.1 even when more $^{45}\text{Ca}^{2+}$ was transversing the vitamin D -replete cells, no more $^{45}\text{Ca}^{2+}$ could be found within these cells than in rachitic cells as there was very little difference in the $^{45}\text{Ca}^{2+}/^{40}\text{Ca}^{2+}$ ratio.

TABLE 8

Analysis of duodenal homogenate at various levels of calcium in the lumen of mucosal cells

The duodenal loop of starved chicks was loaded with 500 μ l $^{45}\text{Ca}^{2+}/^{40}\text{Ca}^{2+}$ containing 0.04 or 2 mg for 2 min or 0.05 or 2 mg for 5 min (1.4.1 and 1.4.2). The loops were removed, washed internally and externally, the mucosal cells were isolated and homogenized (1.5.1 and 1.5.2). The homogenate was extracted for $^{40}\text{Ca}^{2+}$, $^{45}\text{Ca}^{2+}$ and protein (1.7.1) and estimated (1.8). A bone sample was taken from each bird and treated as described in 1.7.4.2. The results expressed as per duodenum are the average for 3 duodena.

TABLE 8

Dose mg	Time min	Type of Chick	Protein mg	$^{40}\text{Ca}^{2+}$ μg	C.P.M.	$^{40}\text{Ca}^{2+}$ /Prot. μg mg	C.P.M./ Prot. mg	C.P.M./ $^{40}\text{Ca}^{2+}$ μg	Bones C.P.M./ whole tibia
0.04	2	Vitamin D ₃ - treated	241	106	946,976	0.43	3,929	8,934	415
	2	Rachitic	202	113	961,217	0.55	4,758	8,506	129
2.0	2	Vitamin D ₃ - treated	231	200	556,506	0.86	2,409	2,783	471
	2	Rachitic	208	206	553,527	0.99	2,661	2,687	478
0.05	5	Vitamin D ₃ - treated	237	136	477,050	0.57	2,013	3,508	1,056
	5	Rachitic	151	96	387,279	0.63	2,565	4,034	348
0.5	5	Vitamin D ₃ - treated	215	153	214,319	0.71	997	1,401	1,789
	5	Rachitic	215	204	357,651	0.94	1,664	1,753	354
2.0	5	Vitamin D ₃ - treated	190	267	354,986	1.40	1,868	1,330	1,303
	5	Rachitic	177	221	258,702	1.24	1,462	1,171	820

PART 2.32.2.3. The "turnover" of calcium in mucosal cells

During Ca^{2+} absorption in vitamin D_3 -replete chicks, approximately 3 to 5 times more $^{45}\text{Ca}^{2+}$ is passing through the cell as compared with rachitic chick, but this is not seen in the $^{45}\text{Ca}^{2+}$ accumulated in the cell as shown in Part 1, Part 2.1 and 2.2. It was observed that there was up to 2.4 times $^{45}\text{Ca}^{2+}$ in the supernatant of vitamin D_3 -replete chicks as compared with rachitic even though the percent $^{45}\text{Ca}^{2+}$ in the supernatant was not more than 5% of the total $^{45}\text{Ca}^{2+}$. In this section an attempt was made to measure the turnover of $^{45}\text{Ca}^{2+}$ during absorption.

METHOD

500 μl $^{45}\text{Ca}^{2+}/^{40}\text{Ca}^{2+}$ solution (0.2 mg, 5 μCi) was placed in the duodenal loop for 8 min then replaced by 500 μl 0.154M-NaCl and absorption allowed to continue for a further 20 min (1.4.3). The cells were isolated, homogenized and fractionated (1.5.1, 1.5.2 and 1.5.3.1). The fractions were treated and estimated for $^{40}\text{Ca}^{2+}$, $^{45}\text{Ca}^{2+}$ and protein (1.7.1 and 1.8).

RESULTS

From control experiments, it was shown that both rachitic and vitamin D_3 -replete duodena accumulated the same amount of $^{45}\text{Ca}^{2+}$ during the 8 min loading period (Table 9). For the next 20 min in the absence of $^{45}\text{Ca}^{2+}$, the vitamin D_3 -replete duodena released more $^{45}\text{Ca}^{2+}$ than did rachitic loops. The results obtained are shown in Table 10 and show that the specific activities of $^{45}\text{Ca}^{2+}$ in the nuclei, mitochondria and microsomes of the vitamin D_3 -

replete cells were less than half those of the rachitic cells.

It was the recently absorbed $^{45}\text{Ca}^{2+}$ that was released from the mucosal cells and the amount of Ca^{2+} per mg protein in the cells was unchanged (Table 10).

TABLE 9

The subcellular distribution of calcium in the duodenal loop during absorption in vivo for 8 minutes

500 μ l $^{45}\text{Ca}^{2+}/^{40}\text{Ca}^{2+}$ solution (0.2 mg, 5 μ Ci) was placed in the duodena of starved chicks left for 8 min and washed externally and internally with warm saline (1.4.1 and 1.4.2). The mucosal cells were isolated (1.5.1), homogenized in 0.25M-sucrose + 2mM-Mg $^{2+}$ (1.5.2) and fractionated (1.5.3.1). A bone sample was taken from each bird and treated as described in 1.7.4.2. The results expressed as per duodenum are the average of 2 experiments with 3 chicks in each group for each experiment. This experiment served as control for Table 10.

Subcellular Fractions	Type of Chick	Protein mg	$^{40}\text{Ca}^{2+}$ μ g	$^{40}\text{Ca}^{2+}$ μ g / Protein mg	C.P.M.	C.P.M./ μ g $^{40}\text{Ca}^{2+}$
Crude Nuclei	Vitamin D $_3$ -treated	66	63	0.96	158,799	2,532
	Rachitic	74	86	1.18	175,279	2,091
Crude Mitochondria	Vitamin D $_3$ -treated	40	49	1.23	155,340	3,189
	Rachitic	35	36	1.03	91,970	2,614
Microsomes	Vitamin D $_3$ -treated	32	14	0.43	22,062	1,570
	Rachitic	31	11	0.35	12,394	1,143
Final	Vitamin D $_3$ -treated	59	26	0.45	12,886	491
	Rachitic	62	19	0.31	8,089	434

The total of protein, $^{40}\text{Ca}^{2+}$, Ca^{45} were for vitamin D $_3$ chick duodenum 198 mg, 152 μ g and 349,087 C.P.M., and for rachitic chick duodenum 203 mg, 152 μ g and 289,732 C.P.M., respectively. The tibia had accumulated in 8 min vitamin D $_3$ chick 7,079 C.P.M./whole tibia and rachitic chick 5,296 C.P.M./whole tibia.

TABLE 10

The "turnover" of calcium absorbed by the duodenum

500 μ l $^{45}\text{Ca}^{2+}/^{40}\text{Ca}^{2+}$ solution (0.2 mg, 5 μ Ci) was placed in the duodenal loop of starved chicks, left for 8 min (1.4.3) and washed out with warm 0.154M-NaCl. 500 μ l 0.154M-NaCl was placed in the loops and left for a further 20 min (1.4.3). Then the chicks were killed and subcellular fractions prepared from the mucosal cells of the duodenum (1.5.1, 1.5.2 and 1.5.3.1). A bone sample was taken from each chick and treated as described in 1.7.4.2. The results are the average of four experiments with 3 chicks in each group for each experiment.

Subcellular Fractions	Type of Chick	Protein mg	$^{40}\text{Ca}^{2+}$ μ g	$^{40}\text{Ca}^{2+}$ μ g / Protein mg	C.P.M.	C.P.M. / μ g $^{40}\text{Ca}^{2+}$
Crude Nuclei	Vitamin D ₃ -treated	58	57	0.99	24,956	424
	Rachitic	64	64	0.99	144,804	2,167
Crude Mitochondria	Vitamin D ₃ -treated	46	47	1.11	31,902	638
	Rachitic	39	38	1.04	88,712	2,179
Microsomes	Vitamin D ₃ -treated	30	13	0.46	5,217	398
	Rachitic	31	12	0.37	10,639	928
Final Supernatant	Vitamin D ₃ -treated	59	2.2	0.04	1,824	980
	Rachitic	60	1.9	0.03	3,708	2,005

$^{45}\text{Ca}^{2+}$ accumulated by tibia for 28 min period were vitamin D₃ chicks, 1,930 C.P.M./whole tibia and rachitic chicks, 1,465 C.P.M./whole tibia.

PART 32.3. The subcellular distribution of calcium and enzymes in the duodenum

The subcellular distribution of calcium in crude fractions was observed in Parts 2.1 and 2.2. Thus in this part an attempt was made to purify the subcellular fractions obtained in parts 2.1 and 2.2. At the same time, purity of these fractions was examined by enzyme assays. Certain enzymes are known to be markers for organelles of the cell. Thus a correlation between Ca^{2+} distribution and the activity of these enzymes was attempted.

METHODS

The duodenal loop was loaded for 20 min with 500 μl $^{45}\text{Ca}^{2+}/^{40}\text{Ca}^{2+}$ solution (2mg, 5 μCi) (1.4.1 and 1.4.2). The mucosal cells were isolated and homogenized (1.5.1 and 1.5.2). The fractionation was carried out as described in 1.5.3.1 and 1.5.3.2, except $2 \times 10^{-5}\text{M}$ chloroquine (Nevaquine, May and Baker) was added to the homogenizing and fractionation media. The fractions were resuspended in 0.25M-sucrose for enzyme assays and then treated as described in 1.7.1, 1.7.2 and 1.8. The enzyme assays were done as described in 1.9. Bone samples were taken and treated as described in 1.7.4.1.

RESULTS

To aid the preservation of membrane structure and to help the yield of the organelles, chloroquine was added to the homogenizing medium of 0.25M-sucrose + 2mM- Mg^{2+} . Weissmann (1968) has reported that chloroquine was useful for preservation of cell

membranes during isolation. This was observed here, as the highest yield, as well as a good separation as shown by $^{45}\text{Ca}^{2+}$ and enzyme distributions (Table 11 and 12).

In Table 11, as observed in most experiments where pure fractions were obtained, at least 60% of the $^{45}\text{Ca}^{2+}$ assayed in the mucosal cells was associated with the mitochondrial fraction. The recovery of $^{45}\text{Ca}^{2+}$ was only about 82%, since $^{45}\text{Ca}^{2+}$ and protein were lost during the purification of nuclei and mitochondria and in some experiments, this $^{45}\text{Ca}^{2+}$ in washing solutions was assayed and accounted for the missing $^{45}\text{Ca}^{2+}$. In three experiments when pure nuclei were isolated in good yield with less than 5% contamination with mitochondria, the nuclear fraction contained between 2 to 10% of the $^{45}\text{Ca}^{2+}$ of the mucosal cell. Therefore it is concluded that the nuclei take up $^{45}\text{Ca}^{2+}$, but in smaller amounts than the mitochondria. Although there was more $^{45}\text{Ca}^{2+}$ being translocated by the vitamin D_3 -repleted chicks, the organelles from these chicks contained slightly less $^{45}\text{Ca}^{2+}$ than the rachitic chicks. The amounts of $^{45}\text{Ca}^{2+}$ in the supernatant of vitamin D_3 -replete chick, as shown earlier, contains nearly 2 times that of rachitic chicks, even though the percent of the total is less than 5%.

The enzyme assays show the the distribution of organelles is fairly well defined as shown in Table 12 and fig. 3. The distribution and total amount of enzyme activities was the same in rachitic and vitamin D_3 -replete chicks, with the exception that the total amount of alkaline phosphatase was increased in the vitamin D -replete group (16 hours after 800 I.U. vitamin D_3), the ratio of

activities being 1.43 ± 0.24 for six experiments. This is in agreement with the findings of Holdsworth (1970). It is known that cytochrome oxidase is a marker for the mitochondria (Reid, 1967). The results show that at least 95% of the cytochrome oxidase activity was recovered in the mitochondrial fraction and only traces were present in the nuclear fraction. Due to the presence of inhibitory material in the original homogenate, the sum of the cytochrome oxidase activities of the fractions was approximately double that of the whole homogenate, but there is no doubt that there is little mitochondrial contamination of either the nuclei or the microsomal fractions. Although mitochondrial contamination of other fraction was small it is clear from an examination of the distribution of the 3 phosphate-hydrolysing enzymes that no fraction was "clean". Acid phosphatase was used to locate the presence of lysosomes (De Duve, Pressman, Gainetto, Wattiaux and Appelmans, 1955; Straus, 1967; Reid, 1967) and the results suggest the nuclei, mitochondria and heavy microsomes fractions contained lysosomes with a preponderance in the heavy microsomes fraction. This distribution is similar to that reported by other workers (De Duve et al, 1955; Gitzelman, Davidson and Osinchak, 1964). The insignificant amount of newly absorbed $^{45}\text{Ca}^{2+}$ in the microsomal fractions suggests that lysosomes are not concerned in calcium transport or have a very small pool which turns over very slowly (Table 11 and 12, and Fig 3).

5' Nucleotidase is believed to be a useful indicator of the presence of plasma membrane in a fraction (Reid, 1967) and results show that

the enzyme was mainly in the nuclear and heavy microsome fractions.

There appears to be no correlation between $^{45}\text{Ca}^{2+}$ and 5' Nucleotidase activity (fig. 3).

TABLE 11

The subcellular distribution of calcium in mucosa of duodenum during absorption in vivo for 20 minutes

500 μ l $^{45}\text{Ca}^{2+}/^{40}\text{Ca}^{2+}$ solution (2 mg, 5 μ Ci) were placed in lumen of starved chicks for 20 min (1.4.1 and 1.4.2). The loop was washed internally and externally before isolation of the mucosal cells (1.5.1). The cells were homogenized in 0.25M-sucrose + 2mM- Mg^{2+} + 2×10^{-5} M-chloroquine and fractionated in media containing 2×10^{-5} M-chloroquine (1.5.3.1 and 1.5.3.2). A bone sample was taken from each chick and treated as described in 1.7.4.1. The results expressed as per duodenum are the average of 12 duodena.

TABLE 11

Fraction	Type of Chick	Protein mg	$^{40}\text{Ca}^{2+}$ μg	C.P.M.	Pi μg	C.P.M./ Ca^{2+} μg
Original Homogenate	Vitamin D ₃ - treated	200	215	125,539	2,657	534
	Rachitic	197	316	208,028	2,415	659
Nuclei	Vitamin D ₃ - treated	14	30	2,683	70	89
	Rachitic	20	42	5,320	45	129
Top band Mitochondria	Vitamin D ₃ - treated	5	12	6,215	8	514
	Rachitic	6	19	5,173	6	271
Middle band Mitochondria	Vitamin D ₃ - treated	18	43	22,524	25	522
	Rachitic	8	56	25,448	19	461
Heavy Mitochondria	Vitamin D ₃ - treated	46	127	71,681	44	568
	Rachitic	67	179	113,462	60	637
Heavy Microsomes	Vitamin D ₃ - treated	15	11	1,263	12	112
	Rachitic	16	11	1,897	14	172
Light Microsomes	Vitamin D ₃ - treated	17	13	1,901	5	147
	Rachitic	11	14	1,617	6	114
Final Supernatant	Vitamin D ₃ - treated	54	18	3,277	366	182
	Rachitic	62	12	1,700	344	141

$^{45}\text{Ca}^{2+}$ accumulated by tibia in 20 min was vitamin D₃ chicks 11,127

C.P.M./tibia (ashed) and rachitic chicks 2,154 C.P.M./tibia (ashed).

TABLE 12

The subcellular distribution of enzymes in mucosa of duodenum

The percent distribution of $^{45}\text{Ca}^{2+}$ is included for comparison. The results are mean values of three separate experiments (total 12 chicks). Rachitic and vitamin D_3 -treated chicks have similar activities except for alkaline phosphatase (see text).

	Cytochrome oxidase	Alkaline phosphatase	Acid phosphatase	5' Nucleotidase	% $^{45}\text{Ca}^{2+}$
Whole homogenate	100	100	100	100	100
Nuclei	9	28	12	31	2
Mitochondria					
Top	2	9	4	3	5
Middle	18	19	9	11	15
Heavy	187	23	18	2	56
Heavy microsomes	3	17	20	29	1
Light microsomes	0	11	12	4	1
Final Supernatant	0	2	13	0	2
% recovery	219*	109	88	80	82

* Some inhibitory material always contributed to the low activity of the whole homogenate.

FIGURE 3

Summary of enzyme assays and ^{45}Ca distribution in subcellular fractions

The histogram shows the percentage of the activity of each enzyme and of $^{45}\text{Ca}^{2+}$ in each fraction. Cytochrome oxidase assay of the fraction was in excess of 2 fold that of the original homogenate.





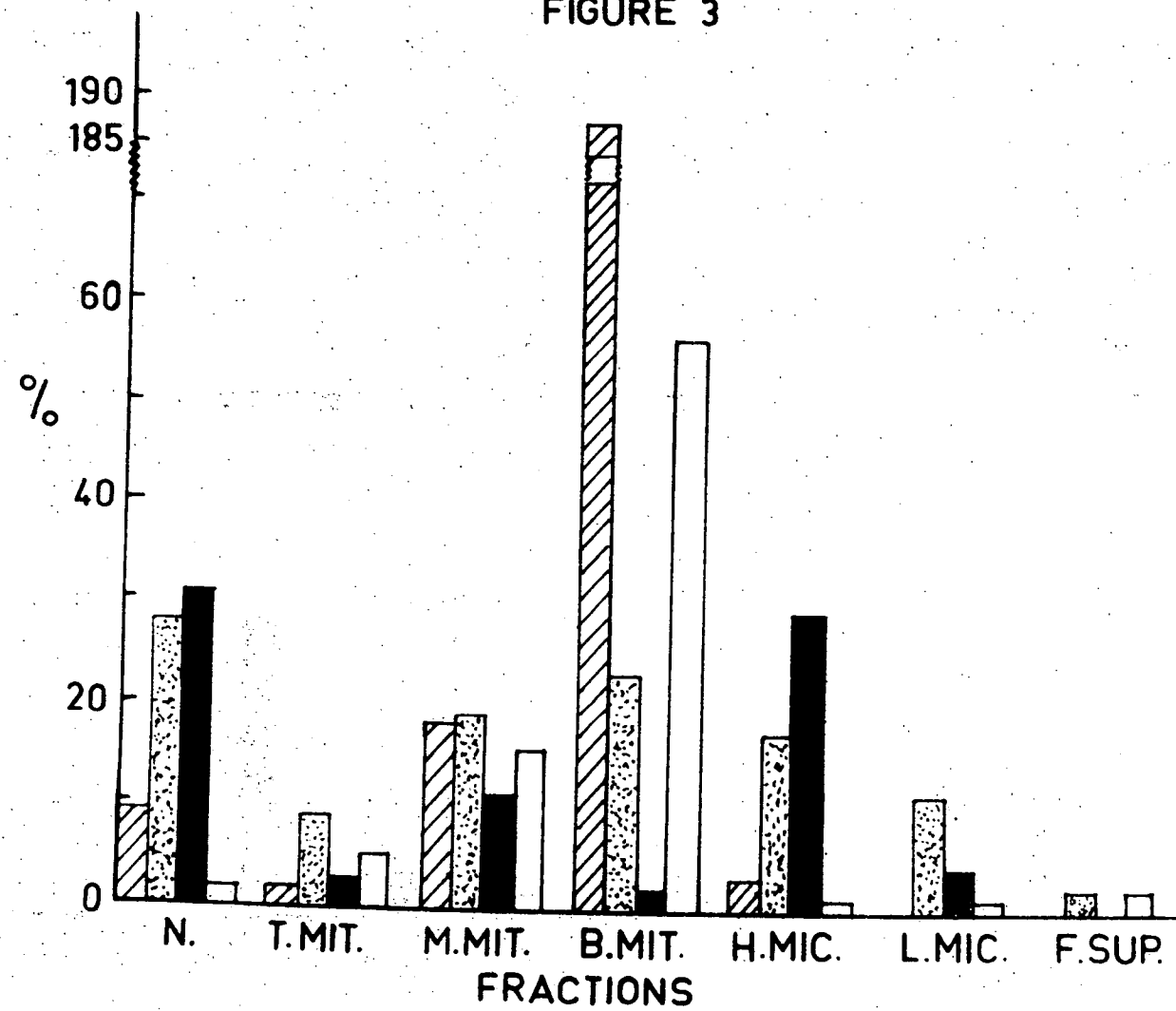
<u>Enzymes</u>		<u>Fractions</u>	
	Cytochrome oxidase	N.	Nuclear
	Alkaline phosphatase	T.MIT.	Top mitochondria
	5' Nucleotidase	M.MIT.	Middle mitochondria
	$^{45}\text{Ca}^{2+}$	B.MIT.	Heavy mitochondria
		H.MIC.	Heavy microsomes
		L.MIC.	Light microsomes
		F.SUP.	Final supernatant

FIGURE 3



DISCUSSION

Many different methods were tried in attempts to obtain complete separation of the subcellular fractions in quantitative yields. Mannitol did not give clean fractions and the use of nonaqueous media gave low yields (Allfrey, Stern, Mirsky and Saetren, 1952). Krebs-Ringer Phosphate buffer (Dawson and Elliott, 1959) containing 0.25M-sucrose or 0.25M-sucrose with 2mM-Mg²⁺, or the latter solution containing 2×10^{-5} M-chloroquine (Nevaquine), all gave similar results when the crude fractionation procedure was used. The medium containing phosphate made the investigation of the distribution and role of inorganic phosphate impossible. Urograffin-glycerol (Williams and Ada, 1967), or dextran in the media interfered with protein estimation. "Tween 80" was used in an attempt to "clean up" the nuclear fraction, but it was found that most of the ⁴⁵Ca²⁺ disappeared from the fraction and was relocated in the final supernatant. It appears that the detergent may have damaged the nuclear membrane, as observed by Stohs and DeLuca (1967) in similar attempts to prepare pure nuclei with media containing Triton X100.

From the initial experiments it was clear that the content of Ca²⁺ in the rachitic and vitamin D₃-replete intestine was the same. The washing out of the gut with various media showed that there was little, if any, effect due to them on the Ca²⁺ content or distribution of these cells.

Differential centrifugation in sucrose solution and analysis of the various fractions for enzyme and for Ca²⁺ showed that at least

60% of the Ca^{2+} in the mucosal cells is associated with mitochondria. When nuclei were carefully prepared, free from mitochondria, they always contained $^{45}\text{Ca}^{2+}$, but the proportion of the total $^{45}\text{Ca}^{2+}$ was 5 to 20%. The heavy and light microsomal fractions also accumulated a small proportion of the total $^{45}\text{Ca}^{2+}$. Acid phosphatase, as a marker for lysosomes (De Duve et al, 1955; Reid, 1967; Straus, 1967), however, could not be correlated with the distribution of $^{45}\text{Ca}^{2+}$ within the organelles. No method was found in which brush borders could be isolated as a separate fraction of the homogenate. Methods for the isolation of brush borders, e.g. Forstner, Sabesin Isselbacher (1968), use media of low ionic strength and containing EDTA and thus preclude the study of the distribution of Ca^{2+} . If it can be assumed that most of the alkaline phosphatase is associated with the brush borders (Hubscher, West and Brindly, 1965), then the fact that alkaline phosphatase was shown not to be associated with the $^{45}\text{Ca}^{2+}$ suggests that little $^{45}\text{Ca}^{2+}$ accumulates at the brush border region. The association of $^{45}\text{Ca}^{2+}$ with mitochondria was observed with a large number of experiments (157), and in these experiments the cytoplasmic Ca^{2+} was always at a very low level. Nevertheless it was noted that the supernatant from vitamin D_3 -repleted chicks generally contained approximately twice the amount of $^{45}\text{Ca}^{2+}$ as the rachitic supernatant.

This association of mitochondria with Ca^{2+} that is being translocated by the intestine is in keeping with the findings of other works examining the endogenous Ca^{2+} in cells. Sampson et al (1970) have reported the presence of granules containing Ca^{2+} in

the mitochondria of rat intestinal mucosa, as observed by electron microscopy. Cassidy, Goldner and Tidball (1969) have shown in mongrel dogs that at least one third of the Ca^{2+} located in the intestine was associated with the mitochondria. Similar results for rat intestine were obtained by Moriuchi et al (1969). These reports concur with distribution of Ca^{2+} within the organelles of the chick mucosal cells obtained here. Other experimenters using liver, beef heart and kidney have also shown a similar distribution of Ca^{2+} in these organelles (Thiers and Vallee, 1957; Drahota et al, 1965; Wester, 1965; Patriarca and Carafoli, 1968). However most published work did not report the purity of the isolated fractions, and the "cell debris" fraction, which contained considerable amounts of Ca^{2+} , apparently would have contained a mixture of cell debris, nuclei and mitochondria.

Since Ca^{2+} on its way through the cell is associated with mitochondria, it was surprising to find that mitochondria from rachitic and vitamin D_3 -replete cells during the absorption of $^{45}\text{Ca}^{2+}$ contained the same amount of $^{45}\text{Ca}^{2+}$, although 3 to 5 times as much $^{45}\text{Ca}^{2+}$ was passing through the replete cells. Some insight into the effect of vitamin D_3 was obtained by studying the "turnover" of $^{45}\text{Ca}^{2+}$ in the intestinal cells. Treatment with vitamin D_3 led to cells that were able to release $^{45}\text{Ca}^{2+}$ more readily than rachitic cells. Since mitochondria appear to contain a large part of the Ca^{2+} in cells and as they as well as other organelles, turnover this Ca^{2+} , it seems that mitochondria could play an important role in the translocation of Ca^{2+} .

There appears to be a slower turnover of Ca^{2+} in the rachitic cell as shown when 2 mg $^{45}\text{Ca}^{2+}$ solution was left in the lumen for 60 min. In these circumstances, the rachitic mucosal cells retain twice as much $^{45}\text{Ca}^{2+}$ as the vitamin D-repleted cells. A similar observation was made during in vivo studies with chick ileal loops (Neville and Holdsworth, 1968).

The distribution reported here was obtained after cooling mucosal cells at 0°C and homogenation in 0.25M-sucrose, a technique used by most research workers in this field. However, it is possible that during this process, a redistribution of Ca^{2+} takes place. The next chapter investigates this problem.

CHAPTER 3THE SUBCELLULAR DISTRIBUTION OF CALCIUM UNDER THE
INFLUENCE OF VARIOUS FACTORSIntroduction

The object of these experiments was to determine whether redistribution of calcium occurred during homogenation and fractionation. A possible reason why the mitochondria contain so much Ca^{2+} is that the mitochondria take up free Ca^{2+} from the cytoplasm during homogenation at 0°C .

In part 1 the uptake of $^{45}\text{Ca}^{2+}$ by the mucosal cell homogenate at 0°C was observed. This study was done to ascertain the distribution of Ca^{2+} within the organelles of the cell homogenate. The uptake of Ca^{2+} by mitochondria at 0°C in the presence and absence of PO_4^{3-} and the effect of pH and Ca^{2+} concentration on the uptake was studied. The release of $^{45}\text{Ca}^{2+}$ from mitochondria was studied in the presence and absence of PO_4^{3-} and at different pH values at 0°C .

In part 2 the effect of inhibitors on uptake and release of Ca^{2+} from mitochondria at 0°C and 30°C was investigated. This study was done to find suitable inhibitors to prevent redistribution of Ca^{2+} within the homogenate.

Some of these inhibitors were then employed in the study of Ca^{2+} uptake by organelles in homogenates of mucosal cells, and on the distribution of Ca^{2+} in homogenates of loaded cells (part 3).

In part 4, an attempt to find out the distribution of Ca^{2+} in the living cell, i.e., at 37°C , was made. Mucosal cells were prepared and homogenized at temperatures above 22°C in the

presence and absence of inhibitors. The usual fractionation procedure used at 0°C would be unsuitable at 30°C, due to the agglutination of the proteins and also to the possibly of the release of Ca^{2+} from organelles at this temperature and from the prolonged times required for fractionation. For this reason a zonal centrifuge, which became available in mid 1972, was used for the fractionation.

PART 13.1 The uptake and release of calcium by cell homogenate and mitochondria

This was a study of the effect of pH and phosphate on uptake of Ca^{2+} by mitochondria at 0°C . The uptake of Ca^{2+} by cell homogenate was observed at 0°C . These studies were done to determine the amount of Ca^{2+} that could be taken up under similar conditions to those present during homogenation.

Part 1.13.1.1. The uptake of calcium by the cell homogenate at 0°C METHOD

A homogenate from non-loaded starved rachitic chick duodenum mucosal cells was prepared as described in 1.5.2. A portion of the homogenate equivalent to half the duodenum mucosal cells was preincubated at 0°C for 5 min before the addition of 1 ml $^{45}\text{Ca}^{2+}/^{40}\text{Ca}^{2+}$ solution (200 $\mu\text{g Ca}^{2+}$, 1 μCi). The homogenate was fractionated as described in 1.5.3.1 and 1.5.3.2.

RESULTS

Of the 200 $\mu\text{g Ca}^{2+}$ added to the homogenate equivalent to half the duodenum, a large proportion was found in the mitochondrial fraction and very little in the final supernatant (Table 13). Thus nearly all the Ca^{2+} was taken up by the organelles, especially the mitochondria, when the equivalent of 400 $\mu\text{g Ca}^{2+}$ was added to one whole duodenum at 0°C .

TABLE 13

The uptake of calcium by the cell homogenate at 0°C

A homogenate from non-loaded starved rachitic chick duodenum mucosal cells was prepared as described in 1.5.2. A portion of the homogenate equivalent to half duodenum was preincubated for 5 min at 0°C before the addition of 1 ml $^{45}\text{Ca}^{2+}/^{40}\text{Ca}^{2+}$ solution (200 μg , 1 μCi). The homogenate was then fractionated as described in 1.5.3.1 and 1.5.3.2. The results are expressed as per whole duodenum.

Fraction	Protein mg	$^{40}\text{Ca}^{2+}$ μg	C.P.M.	C.P.M./ $^{40}\text{Ca}^{2+}$ μg
Original homogenate	214	224	-	-
Crude nuclei	60	150	348,800	2,290
Crude mitochondria	86	496	1,318,374	2,658
Heavy microsomes	14	8	7,884	889
Light microsomes	18	12	3,104	247
Final supernatant	80	26	6,980	278

Part 1.23.1.2. The effect of pH and phosphate on mitochondrial calcium uptake and release at 0°C and uptake of calcium by mitochondria at various concentrations of calciumMETHODS

Mitochondrial suspensions were prepared from duodena of non-loaded or $^{45}\text{Ca}^{2+}$ loaded starved rachitic chicks as described in

1.5.3.3. The mitochondria were resuspended in 0.25M-sucrose + 50mM-Tris Tricine at the appropriate pH and adjusted to contain between 4 to 7 mg/ml. For the uptake experiments, from 2.5 μg to 20.0 $\mu\text{g/ml}$ $^{45}\text{Ca}^{2+}/^{40}\text{Ca}^{2+}$ solution were added to 1 ml of mitochondrial suspension at 0°C. Before the addition of $^{45}\text{Ca}^{2+}$, 0.22 $\mu\text{moles PO}_4^{3-}$ was added to some tubes. A sample was taken at 15 min and filtered through millipore filter as described in 1.6.2.2. The results are calculated as a percent of $^{45}\text{Ca}^{2+}$ taken up by the mitochondria.

The release of $^{45}\text{Ca}^{2+}$ from mitochondria was determined by adding at time zero, 0.22 $\mu\text{moles PO}_4^{3-}$. A sample was taken at 15 min. The sample was filtered through a millipore filter as described in 1.6.2.2. The results being expressed as a percent release of total $^{45}\text{Ca}^{2+}$.

RESULTS

At 0°C the maximum uptake of Ca^{2+} occurred at pH 6.5 in the presence of PO_4^{3-} . The uptake decreased as the pH rose from pH 6.5 to 7.3 although uptake was always greater in the presence of PO_4^{3-} (Table 14).

There was very little release at pH 6.5 after 15 min and the release only slightly increased from pH 6.5 to 7.3. Phosphate

appeared to have little effect on the release of Ca^{2+} from mitochondria. Mitochondria from both rachitic and vitamin D-replete chicks appear to take up similar amounts of Ca^{2+} at pH 7.2 at 0°C for 15 min. The maximum level reached being $2.07 \mu\text{g } ^{45}\text{Ca}^{2+}/\text{mg}$ protein for vitamin D treated chick mitochondria and $1.6 \mu\text{g } ^{45}\text{Ca}^{2+}/\text{mg}$ protein for rachitic chick mitochondria (Table 15). The amount of $2 \mu\text{g } \text{Ca}^{2+}$ accumulated per mg mitochondrial protein is equivalent to 50 nmoles/mg and thus corresponds to limited loading of mitochondria. Similarly Chance (1963; 1965) and Rossi and Lehninger (1963) showed that under limited loading conditions, mitochondria could take up to 100 nmoles/mg.

TABLE 14

The effect of pH and phosphate on mitochondrial calcium uptake and release at 0°C

The mitochondria were prepared from rachitic chick duodenum mucosal cells that had or had not been loaded with $^{45}\text{Ca}^{2+}$ as described in 1.5.3.3. The mitochondria were resuspended in 0.25M-sucrose + 50mM-Tris Tricine at the appropriate pH.

The mitochondrial suspension for the uptake experiment contained 6.3 mg protein/ml. To 1 ml of the mitochondrial suspension was added 0.22 $\mu\text{moles PO}_4^{3-}$ and 25 $\mu\text{l } ^{45}\text{Ca}^{2+}/^{40}\text{Ca}^{2+}$ solution (5 μg , 0.25 μCi) at time zero.

The mitochondrial suspension for release experiment contained 5.5 mg protein/ml. When PO_4^{3-} was added, 0.22 μmoles were added to 1 ml of mitochondrial suspension.

A sample was taken after 15 min incubation at 0°C and filtered through millipore filter as described in 1.6.2.2. The results are the average of duplicate determinations expressed as % of total $^{45}\text{Ca}^{2+}$ taken up or % of total $^{45}\text{Ca}^{2+}$ released from mitochondria.

pH	PO_4^{3-} μmoles added	% uptake of $^{45}\text{Ca}^{2+}$	% release of $^{45}\text{Ca}^{2+}$
6.5	none	73	1
	0.22	84	0
7.0	none	64	2
	0.22	76	1
7.3	none	48	4
	0.22	58	6

TABLE 15

The effect of various calcium concentrations on uptake by mitochondria at 0°C

The mitochondria were prepared as described in 1.5.3.3. from starved rachitic and vitamin D₃-replete chick duodenum mucosal cells. The mitochondria were suspended in 0.25M-sucrose + 50mM-Tris Tricine pH 7.2. The suspensions contained 4.8 mg protein/ml for rachitic and 5.1 mg protein/ml for vitamin D₃ mitochondria.

To 1 ml of mitochondrial suspension in 0.25M-sucrose + 50mM-Tris Tricine pH 7.2 was added 100 μ l $^{45}\text{Ca}^{2+}/^{40}\text{Ca}^{2+}$ solution ($X \mu\text{g}$, 0.1 μCi) at 0°C. A sample was taken after 15 min and filtered through millipore as described in 1.6.2.2. The results are the average for 2 experiments each with duplicate determinations expressed as % of total $^{45}\text{Ca}^{2+}$ accumulated by the mitochondria.

Type of mitochondria	amount of $^{45}\text{Ca}^{2+}/^{40}\text{Ca}^{2+}$ added μg	% $^{45}\text{Ca}^{2+}$ taken up	amount of $^{45}\text{Ca}^{2+}/^{40}\text{Ca}^{2+}$ $\mu\text{g}/\text{mg}$ protein
Vitamin D ₃ treated	2.5	84	0.50
	5.0	72	0.79
	10.0	55	1.23
	20.0	42	2.07
Rachitic	2.5	68	0.36
	5.0	65	0.71
	10.0	57	1.21
	20.0	37	1.69

PART 23.2 The effect of inhibitors on calcium uptake by mitochondria

In part 1 it was determined that isolated mitochondria and the homogenate were able to take up large amounts of Ca^{2+} . The experiments done in this part were to ascertain a suitable combination of inhibitors to prevent Ca^{2+} redistribution if it occurs during homogenation of the microsomal cells at 0°C and 30°C .

3.2.1 The effect of inhibitors on uptake and release of calcium by mitochondriaMETHODS

Mitochondrial suspensions were prepared in 0.25M-sucrose + 50mM-Tris Tricine pH 6.5 - 7.1 as described in 1.5.3.3 and were adjusted to contain between 3 and 6 mg protein/ml.

For the uptake of $^{45}\text{Ca}^{2+}$ by mitochondria they were preincubated for 5 min with the inhibitor at 0°C or 30°C before the addition of 25 μl $^{45}\text{Ca}^{2+}/^{40}\text{Ca}^{2+}$ solution (5 μg , 0.1 μCi) After 15 min a sample was filtered through millipore membranes described in 1.6.2.2. The uptake of $^{45}\text{Ca}^{2+}$ was expressed as a percentage of total $^{45}\text{Ca}^{2+}$.

The release of $^{45}\text{Ca}^{2+}$ from mitochondria was studied in the presence of inhibitors, by adding 1 ml of mitochondrial suspension ($^{45}\text{Ca}^{2+}$ loaded) containing 3 - 6 mg protein/ml to 1 ml 0.25M-sucrose with or without inhibitor or 1 ml 0.25M-sucrose + 31mM-imidazole + 28mM-succinate pH 7.1 + 10 mg BSA with or without inhibitors. This mixture was incubated for 15 min at 0°C or 30°C . A sample was centrifuged or filtered through millipore filter at 15 min as described

in 1.6.2.1 or 1.6.2.2. The results being calculated as percent $^{45}\text{Ca}^{2+}$ released from the mitochondria as of the total $^{45}\text{Ca}^{2+}$.

RESULTS

3.2.1.1 The effect of inhibitors on uptake of calcium by mitochondria at 0°C and 30°C.

From Table 16 one can see in buffered sucrose that dicoumarol, 2,4 DNP, EGTA, La^{3+} and ruthenium red as well as NaF and antimycin + warfarin in unbuffered sucrose inhibited $^{45}\text{Ca}^{2+}$ uptake by 20% or greater. In succinate medium antimycin, dicoumarol, 2,4 DNP, N-ethyl maleimide, warfarin + antimycin and EGTA, inhibited $^{45}\text{Ca}^{2+}$ uptake by mitochondria.

Ruthenium red was found to inhibit the uptake of $^{45}\text{Ca}^{2+}$ by mitochondria incubated at 30°C in succinate and sucrose media (Table 17). The inhibition was about the same as obtained at 0°C.

3.2.1.2 The effect of inhibitors on the release of calcium by mitochondria

From Table 18, it was ascertained that gramicidin, dicoumarol, 2,4 DNP, antimycin and antimycin + warfarin caused release of $^{45}\text{Ca}^{2+}$ from mitochondria that was greater than 20%. The other inhibitors, NaF, warfarin, N-ethyl maleimide, EGTA, and ruthenium red did not cause release of $^{45}\text{Ca}^{2+}$ in buffered sucrose or succinate media at 0°C.

At 30°C in sucrose medium ruthenium red did not cause release of $^{45}\text{Ca}^{2+}$ from mitochondria (Table 19A). While EDTA at concentration of 10^{-4}M caused about 23% $^{45}\text{Ca}^{2+}$ release from $^{45}\text{Ca}^{2+}$ loaded mitochondria in the 15 min period (Table 19B).

TABLE 16

The effect of inhibitors on uptake of calcium by mitochondria at 0°C

A mitochondrial suspension was prepared from starved rachitic chick duodenum mucosal cells in 0.25M-sucrose + 50mM-Tris Tricine pH 6.5 as described in 1.5.3.3. The mitochondrial suspension contained 4.0 mg protein/ml. 1 ml of the mitochondrial suspension was preincubated at 0°C for 5 min in media containing the inhibitor before the addition of 25 μ l $^{45}\text{Ca}^{2+}/^{40}\text{Ca}^{2+}$ solution (5 μ g, 0.1 μ Ci) (1.6.2.2). After 15 min a sample was centrifuged (1.6.2.1) or was filtered through millipore membrane as described in 1.6.2.2. The uptake of $^{45}\text{Ca}^{2+}$, the average of duplicate determination, are expressed as % of the total $^{45}\text{Ca}^{2+}$.

Inhibitor	final conc M	% inhibition of uptake in buffered sucrose pH 6.5	% inhibition of uptake in succinate medium pH 7.1
none		0	0
antimycin	10^{-5}	-	68
gramicidin	10^{-5}	0	7
dicoumarol	10^{-4}	39	72
2,4 Dinitrophenol	10^{-4}	22	74
sodium fluoride	10^{-2}	66 ^x	-
warfarin	10^{-4}	14	19
N-ethyl maleimide	10^{-3}	16	58
antimycin	10^{-5}		
+ warfarin	10^{-4}	64 ^x	69
EGTA	10^{-3}	75	74
rotenone	10^{-4}	13	-
rotenone	10^{-4}		
+ warfarin	10^{-4}	19	-
La^{3+}	10^{-4}	28	-
ruthenium red	0.7 mg/ml	64	-

x in buffered sucrose

- not done

TABLE 17

The effect of ruthenium red on uptake of calcium by mitochondria at 30°C

A mitochondrial suspension was prepared from starved rachitic chick duodenum mucosal cells in 0.25M-sucrose + 50mM-Tris Tricine pH 7.1 as described in 1.5.3.3. The mitochondrial suspension contained 3.5 mg protein/ml. 1 ml of the suspension was preincubated at 30°C for 5 min in 1 ml 0.25M-sucrose + 50mM-Tris Tricine pH 7.1 + 10 mg BSA + 1.4 mg ruthenium red or 1.0 ml 0.25M-sucrose + 31mM-imidazole + 28mM-succinate pH 7.1 + 10 mg BSA + 2.8 mg ruthenium red before the addition of 25 μ l $^{45}\text{Ca}^{2+}/^{40}\text{Ca}^{2+}$ solution (5 μ g, 0.1 μ Cl). A sample was filtered through millipore filter as described in 1.6.2.2. The results are the average for 2 experiments with duplicate determinations expressed as a percent of $^{45}\text{Ca}^{2+}$ taken up.

Inhibitor	buffered sucrose pH 7.1		succinate medium pH 7.1	
	% $^{45}\text{Ca}^{2+}$ taken up	% inhibition of uptake	% $^{45}\text{Ca}^{2+}$ taken up	% inhibition of uptake
none	82	0*	89	0*
ruthenium red	9	73	14	75

* assumed as counted

TABLE 18

The release of calcium by mitochondria in the presence of inhibitors at 0°C

The duodenal loops of starved rachitic chicks were loaded with $^{45}\text{Ca}^{2+}$ as described in 1.4.2 and the mucosal cells were isolated as described in 1.5.1, 1.5.2 and 1.5.3.3. A mitochondrial suspension was prepared in 0.25M-sucrose or 0.25M-sucrose + 50mM-Tris Tricine pH 6.5. This suspension contained 4.0 mg protein/ml. The mitochondria were incubated in 0.25M-sucrose with or without inhibitor or 0.25M-sucrose + 15.5mM-imidazole + 14mM-succinate pH 7.1 with or without inhibitor for 15 min at 0°C. A sample was taken at 15 min from the mixture and centrifuged as described in 1.6.2.1 or filtered as described in 1.6.2.2. The results are the average of 2 experiments with duplicate determinations expressed as the percent release of total $^{45}\text{Ca}^{2+}$ content of the system.

TABLE 18

Inhibitors	final conc M	% release of $^{45}\text{Ca}^{2+}$ in unbuffered sucrose	% release of $^{45}\text{Ca}^{2+}$ in succinate medium pH 7.1
none		2	2
antimycin	10^{-5}	2	22
gramicidin	10^{-5}	22	4
dicoumarol	10^{-4}	49	45
2,4 Dinitrophenol	10^{-4}	65	46
sodium fluoride	10^{-2}	2	-
warfarin	10^{-4}	2	5
N-ethyl maleimide	10^{-3}	4	10
antimycin + warfarin	10^{-5} 10^{-4}	8	22
EGTA	10^{-3}	5 ^x	4
rotenone	10^{-4}	5 ^x	-
rotenone + warfarin	10^{-4} 10^{-4}	7 ^x	-
ruthenium red	0.7 mg/ml	3 ^x	-

x done in buffered sucrose pH 7.0

- not done

TABLE 19A

The effect of ruthenium red on the release of calcium from mitochondria at 30°C

Mitochondria were prepared from preloaded $^{45}\text{Ca}^{2+}$ mucosal cells of duodenum of starved rachitic chick as described in 1.4.1, 1.4.2 and 1.5.3.3. The mitochondrial suspension contained 3.5 mg protein/ml in 0.25M-sucrose + 50mM-Tris Tricine pH 7.1. 1 ml of the suspension was incubated with 1 ml 0.25M-sucrose with and without 1.4 mg ruthenium red at 30°C for 15 min. A sample was filtered through millipore filter as described in 1.6.2.2. The results are expressed as the average of 2 experiments each with duplicate determinations.

Inhibitor	% release of $^{45}\text{Ca}^{2+}$
none	12
ruthenium red (0.7 mg/ml)	13

TABLE 19B

The effect of EDTA on release of calcium from mitochondria at 30°C

Mitochondria were prepared from preloaded $^{45}\text{Ca}^{2+}$ mucosal cells of duodenum of starved rachitic chick as described in 1.4.1, 1.4.2 and 1.5.3.3. The mitochondrial suspension contained 3.5 mg protein/ml in 0.25M-sucrose. 1 ml of the suspension was incubated with 1 ml 0.25M-sucrose with or without 2×10^{-4} M-EDTA for 15 min at 30°C. A sample was filtered through millipore filter as described in 1.6.2.2. The results are the average of duplicate determinations expressed as % release of $^{45}\text{Ca}^{2+}$ from the mitochondria.

Inhibitor	% release of $^{45}\text{Ca}^{2+}$
none	4
EDTA (10^{-4} M)	23

PART 33.3 The effect of inhibitors on the subcellular distribution of calcium in the mucosal cell

In 3.2, a number of inhibitors were studied for their effects on uptake and release of Ca^{2+} by mitochondria. From these studies dicoumarol, antimycin plus warfarin and later rotenone plus warfarin (due to the unavailability of antimycin in Australia), EGTA, sodium fluoride and ruthenium red were further examined. La^{3+} was not used because it would precipitate in the presence of phosphate.

In part 3.1, the effect of these inhibitors on the uptake of $^{45}\text{Ca}^{2+}$ by the whole homogenate was studied and in 3.3.2, the effect of the inhibitors on the distribution of $^{45}\text{Ca}^{2+}$ in previously loaded mucosal cells was examined.

Part 3.13.3.1 The in vitro effect of inhibitors on calcium uptake by cell homogenate3.3.1.1 The effect of dicoumarol on uptake of calcium by the cell homogenate at 0°CMETHODS

3.3.1.1.1 Duodenum mucosal cells from 4 starved, non-loaded rachitic chicks were isolated and prepared as a homogenate as described in 1.5.1 and 1.5.3. The homogenate was prepared in Krebs-Ringer phosphate + 0.25M-sucrose. 5 ml of the homogenate was added to 5 ml Krebs-Ringer phosphate + 0.25M-sucrose with or without $2 \times 10^{-4}\text{M}$ -dicoumarol and stirred for 10 min at 0°C. To one set of mixtures was added 50 μl $^{45}\text{Ca}^{2+}/^{40}\text{Ca}^{2+}$ solution (200 μg , 0.5 μCi) and stirred for a further 20 min. The mixtures were layered over 0.34M-sucrose with or without 10^{-4}M -dicoumarol and fractionated as described in 1.5.3.1 and 1.5.3.2.

3.3.1.1.2 Duodenum mucosal cells from 2 starved rachitic and 2 starved vitamin D₃-replete chicks were isolated as described in 1.5.1. The cells were homogenized in Krebs-Ringer phosphate + 0.25M-sucrose with or without 10⁻⁴M-dicoumarol as described 1.5.2. The homogenate was centrifuged at 600g for 15 min. The residue was re-homogenized and added to the supernatant. To 10 ml of the homogenate was added 50 μ l ⁴⁵Ca²⁺/⁴⁰Ca²⁺ solution (200 μ g, 0.5 μ Cl) and stirred for 20 min. The mixture was layered over 0.34M-sucrose with or without 10⁻⁴M-sucrose and fractionated as described in 1.5.3.1 and 1.5.3.2.

RESULTS

3.3.1.1.1 and 3.3.1.1.2 Dicoumarol, as shown in Table 20 and 21, inhibits the uptake by mitochondrial and nuclear fractions. The ⁴⁵Ca²⁺ not taken up by these fractions was mainly in the cytoplasmic fraction. This inhibitor caused the release of some of the endogenous Ca²⁺ in the nuclear and mitochondrial fractions and this Ca²⁺ became associated with microsomes (Table 20). However the release of endogenous Ca²⁺, amounted to about 23%. Dicoumarol reduced ⁴⁵Ca²⁺ binding or uptake by the mitochondrial fraction by about 70% and nuclear fraction by about 90%.

TABLE 20

The effect of dicoumarol on calcium distribution in the cell homogenate at 0°C

Duodenum mucosal cells from 4 starved, non-loaded rachitic chicks were isolated and prepared as a homogenate as described in 1.5.1 and 1.5.2. The homogenate was prepared in Krebs-Ringer phosphate + 0.25M-sucrose. 5 ml of the homogenate was added to 5 ml Krebs-Ringer phosphate + 0.25M-sucrose with or without 2×10^{-4} M dicoumarol and stirred for 10 min at 0°C. To one set of mixtures was added 50 μ l $^{45}\text{Ca}^{2+}/^{40}\text{Ca}^{2+}$ solution (200 μ g, 0.5 μ Ci) and stirred for a further 20 min. The mixtures were layered over 0.34M-sucrose with or without 10^{-4} M-dicoumarol and fractionated as described in 1.5.3.1 and 1.5.3.2. The results are expressed as per 5 ml of the original homogenate per duodenum.

Table 20

Fraction	$^{45}\text{Ca}^{2+}$ added	protein mg		$^{40}\text{Ca}^{2+}$ μg		C.P.M.		C.P.M./ $^{40}\text{Ca}^{2+}$ μg	
		-*	+*	-	+	-	+	-	+
original homogenate	none	211		148					
crude nuclei	none	45	48	45	30	-	-	-	-
	200 μg	41	49	110	33	45,098	5,796	406	170
crude mitochondria	none	59	60	79	44	-	-	-	-
	200 μg	59	56	232	117	90,690	44,266	390	379
heavy microsomes	none	10	12	11	18	-	-	-	-
	200 μg	11	9	9	15	791	4,095	87	273
light microsomes	none	14	10	9	14	-	-	-	-
	200 μg	14	14	9	20	730	8,407	79	410
final supernatant	none	60	56	2	4	-	-	-	-
	200 μg	63	68	3	151	99	69,010	29	456

* - = Fractionation medium without dicoumarol

* + = Fractionation medium with dicoumarol

Table 21

Fraction	Type of chick	protein mg		$^{40}\text{Ca}^{2+}$ μg		C.P.M.		C.P.M. $^{40}\text{Ca}^{2+}$ μg	
		-*	+*	-	+	-	+	-	+
original homogenate	Vitamin D ₃ -treated	173	178	79	90	-	-	-	-
	Rachitic	155	154	66	72	-	-	-	-
crude nuclei	Vitamin D ₃ -treated	43	39	120	27	53,509	2,828	447	104
	Rachitic	38	36	123	16	53,599	1,299	435	81
crude mitochondria	Vitamin D ₃ -treated	50	42	160	49	83,353	19,532	519	395
	Rachitic	49	45	154	42	85,799	16,535	557	391
heavy microsomes	Vitamin D ₃ -treated	11	9	5	14	1,659	6,732	317	474
	Rachitic	12	9	5	15	1,153	6,579	228	427
light microsomes	Vitamin D ₃ -treated	13	12	14	27	1,334	9,353	98	352
	Rachitic	10	11	12	25	929	9,905	81	396
final supernatant	Vitamin D ₃ -treated	66	61	3	224	671	99,410	210	445
	Rachitic	60	59	4	209	384	106,212	98	508

* - = Fractionation medium without dicoumarol

* + = Fractionation medium with dicoumarol

TABLE 21

The effect of dicoumarol on calcium distribution in the cell homogenate at 0°C

Mucosal cells from 2 starved rachitic and 2 starved vitamin D₃-replete chicks duodenum were isolated as described in 1.5.1. The cells were homogenized in Krebs-Ringer phosphate with or without 10^{-4} M dicoumarol + 0.25M-sucrose as described in 1.5.2. The homogenate was centrifuged at 600g for 15 min. The residue was rehomogenized and added to supernatant. To 10 ml of homogenate was added 50 μ l $^{45}\text{Ca}^{2+}/^{40}\text{Ca}^{2+}$ solution (200 μ g, 0.5 μ Ci) and stirred for 20 min. The mixture was layered over 0.34M-sucrose with or without 10^{-4} M-dicoumarol and fractionated as described in 1.5.3.1 and 1.5.3.2. The results are the average of duplicate determinations expressed as per duodenum (10 ml of homogenate).

3.3.1.2 The effect of antimycin plus warfarin and of EGTA on calcium uptake by the cell homogenate at 0°C

METHODS

3.3.1.2.1. A homogenate of non-loaded duodenum mucosal cells from 3 rachitic chicks was prepared as described in 1.6.1. The homogenate was centrifuged at 600g, the residue rehomogenized and added to supernatant. 10 ml of the homogenate was preincubated in 10 ml 0.25M-sucrose + 50mM-Tris-Tricine pH 7.1 with or without inhibitors for 10 min at 0°C. Then 200 μ l $^{45}\text{Ca}^{2+}/^{40}\text{Ca}^{2+}$ solution (400 μ g, 2 μ Ci) was added and stirred 5 min. The mixtures were layered over 0.34M-sucrose with or without inhibitors and then fractionated as described in 1.5.3.1 and 1.5.3.2.

3.3.1.2.2. The duodenal loops of rachitic chicks were loaded with $^{40}\text{Ca}^{2+}$ for 20 min as described in 1.4.1 and 1.4.2. The mucosal cells were isolated, homogenized and centrifuged at 600g for 10 min. The pellet was rehomogenized and added to the supernatant as described in 1.5.1 and 1.5.2. To this homogenate with stirring for 1 min was added 50 μ l $^{45}\text{Ca}^{2+}/^{40}\text{Ca}^{2+}$ solution (60 μ g, 0.5 μ Ci). 10 ml of the $^{45}\text{Ca}^{2+} + ^{40}\text{Ca}^{2+}$ loaded homogenate was mixed for 5 min at 0°C with 10 ml 0.25M-sucrose with or without inhibitors. These mixtures were then fractionated as described in 1.5.3.1 and 1.5.3.

RESULTS

3.3.1.2.1 and 3.3.1.2.2. The effect of EGTA on Ca^{2+} uptake by the homogenate was to reduce the $^{45}\text{Ca}^{2+}$ uptake by both nuclei and mitochondria as shown by their lower $^{40}\text{Ca}^{2+}$, $^{45}\text{Ca}^{2+}$ and specific activities (Table 22). The inhibition of uptake was not complete.

Again, as was seen when using dicoumarol, the microsomal fractions did in fact take up more of the $^{45}\text{Ca}^{2+}$ than the control.

Antimycin and warfarin inhibited Ca^{2+} uptake to limited extent in this homogenate (Table 22). However the inhibition appears to be different to that shown for dicoumarol and EGTA, in that the nuclear $^{45}\text{Ca}^{2+}$ content was markedly decreased while the mitochondrial $^{45}\text{Ca}^{2+}$ content and specific activity was only slightly decreased. This combination of inhibitors did not cause a great release of endogenous Ca^{2+} from the organelles as shown by the specific activity of the cytoplasmic fraction.

However, to test this point more directly, a homogenate from cells previously loaded with stable Ca^{2+} and then with $^{45}\text{Ca}^{2+}$ at 0°C was treated with either sucrose only or sucrose containing these inhibitors. It can be seen from Table 23 that antimycin plus warfarin caused only slight release of $^{45}\text{Ca}^{2+}$, and EGTA caused a maximum of 13% release of $^{45}\text{Ca}^{2+}$ which appeared in the final supernatant. It was of interest that cells loaded in vivo with a large amount of Ca^{2+} , when homogenized in sucrose at 0°C , still took up extra $^{45}\text{Ca}^{2+}$ completely (cf. with control column of Table 23).

TABLE 22

The effect of antimycin plus warfarin and of EGTA on uptake of calcium by the homogenate at 0°C

A homogenate of non-loaded duodenum mucosal cells from 3 starved rachitic chicks was prepared as described in 1.6.1. The homogenate was centrifuged, the residue rehomogenized and added to the supernatant. 10 ml of homogenate was preincubated in 10 ml 0.25M-sucrose + 50mM-Tris Tricine pH 7.1 with or without inhibitors for 10 min at 0°C. Then 200 μ l $^{45}\text{Ca}^{2+}/^{40}\text{Ca}^{2+}$ solution (400 μ g, 2 μ Ci) was added with stirring for 5 min. The mixtures were layered over 0.34M-sucrose with or without inhibitors and then fractionated as described in 1.5.3.1 and 1.5.3.2. The inhibitors were used at the following final concentration; 10^{-5} M-antimycin + 10^{-4} M-warfarin or 10^{-3} M-EGTA. The results are the average of duplicate determinations and are expressed as per duodenum (10 ml $\hat{=}$ duodenum).

TABLE 22

	protein mg			$^{40}\text{Ca}^{2+}$ μg				C.P.M.			C.P.M./ $^{40}\text{Ca}^{2+}$ μg	
	-	A+W	EGTA*	-	A+W	EGTA	-	A+W	EGTA	-	A+W	EGTA
original homogenate	255			205								
crude nuclei	60	67	74	147	71	44	348,800	98,574	16,136	2,290	1,386	430
crude mitochondria	87	97	88	497	414	272	1,317,861	929,479	235,494	2,651	2,245	866
heavy microsomes	15	14	14	9	19	12	7,884	38,374	20,658	889	2,042	1,723
light microsomes	18	15	14	13	22	10	3,104	61,308	17,462	247	2,758	1,746
final supernatant	79	77	71	27	157	424	6,980	535,461	1,381,682	278	3,410	3,268

* A+W = Fractionation medium containing antimycin plus warfarin

* EGTA = Fractionation medium containing EGTA

* - = Fractionation medium containing no inhibitor

TABLE 23

The effect of antimycin plus warfarin and of EGTA on the ^{45}Ca Calcium distribution in ^{40}Ca Calcium loaded mucosal cell

The duodenal loop of rachitic chicks were loaded with $^{40}\text{Ca}^{2+}$ for 20 min as described in 1.4.1 and 1.4.2. The mucosal cells were isolated, homogenized and centrifuged at 800g for 10 min. The pellet was rehomogenized and added to the supernatant as described in 1.5.1 and 1.5.2. To this homogenate, with stirring for 1 min, was added 50 μl $^{45}\text{Ca}^{2+}/^{40}\text{Ca}^{2+}$ solution (60 μg , 0.5 μCi). 10 ml of the $^{45}\text{Ca}^{2+}/^{40}\text{Ca}^{2+}$ loaded homogenate was mixed for 5 min at 0°C with 10 ml 0.25M-sucrose with or without inhibitors. These mixtures were then fractionated as described in 1.5.3.1 and 1.5.3.2. The final concentration of the inhibitors used in the homogenizing and fractionation media was: 10^{-5}M -antimycin plus 10^{-4}M -warfarin or 10^{-3}M -EGTA. The results are for a single experiment as expressed as per duodenum.

TABLE 23

	protein mg	40Ca ²⁺ μg	C.P.M. A+W	EGTA	C.P.M. A+W	EGTA	C.P.M. 40Ca ²⁺ μg
	* A+W	* A+W	* A+W	* A+W	* A+W	* A+W	* A+W
original homogenate	211	397					
60 μg 45Ca ²⁺ /40Ca ²⁺	211	467	88,858				190
crude nuclei	59	61	66	256	234	37,343	37,897
crude mitochondria	39	41	43	168	131	43,016	40,366
heavy microsomes	9	10	12	7	9	1,167	697
light microsomes	19	12	13	10	7	107	29
final supernatant	49	49	48	42	34	68	0
							1,115
							11,165
							0
							33
							163

* A + W = Fractionation medium containing antimycin plus warfarin

* EGTA = Fractionation medium containing EGTA

* - = Fractionation medium containing no inhibitor

3.3.1.3 The effect of ruthenium red on calcium uptake by the cell homogenate at 0°C

METHOD

A homogenate from duodenum mucosal cells of starved rachitic chick was prepared as described in 1.6.1.1 in 0.25M-sucrose + 2mM-Mg²⁺ + 50mM-Tris-Tricine pH 7.2. To 8 ml of the homogenate was added 8 ml 0.25M-sucrose + 2mM-Mg²⁺ + 50mM-Tris-Tricine with or without 11.2 mg ruthenium red and stirred at 0°C for 10 min. Then 250 μ l ⁴⁵Ca²⁺/⁴⁰Ca²⁺ (50 μ g, 0.5 μ Ci) were added with stirring for 5 min. The homogenate was layered over 0.34M-sucrose with or without 1.4 mg/ml ruthenium red and fractionated as described in 1.5.3.1 and 1.5.3.2.

RESULTS

Ruthenium red as previously shown in 3.2 inhibited Ca²⁺ uptake by mitochondria at 0°C while it did not cause release. It had a profound effect on the organelles present in the homogenate. A considerable amount of the dye was bound to the organelles and protein, and disturbed the usual pattern obtained on centrifugation, causing a large portion to precipitate at low centrifugal force. It caused approximately 80% inhibition of uptake of ⁴⁵Ca²⁺ particularly inhibiting uptake by nuclei and mitochondria. The cytoplasmic fraction contained 100% ⁴⁰Ca²⁺ and 80% ⁴⁵Ca²⁺ that was added to the homogenate (Table 24).

TABLE 24

The effect of ruthenium red on calcium uptake by the cell homogenate at 0°C

A homogenate was prepared from duodenum mucosal cells of 3 starved rachitic chicks as described in 1.5.1 and 1.5.2. 8 ml of the homogenate was added to 8 ml 0.25M-sucrose + 50mM-Tris Tricine Ph 7.2 with or without 11.2 mg ruthenium red and was preincubated at 0°C for 10 min. Then 250 μ l $^{45}\text{Ca}^{2+}/^{40}\text{Ca}^{2+}$ solution (50 μ g, 0.5 μ Ci) were added and further incubated at 0°C for 5 min with stirring. The homogenate was layered over 0.34M-sucrose with or without 1.4 mg/ml ruthenium red and then fractionated as described in 1.5.3.1 and 1.5.3.2. The results are the average of duplicate determinations expressed as per duodenum.

Fraction	protein mg		$^{40}\text{Ca}^{2+}$ μ g		C.P.M.		C.P.M./ $^{40}\text{Ca}^{2+}$ μ g	
	-	+	-	+	-	+	-	+
original homogenate	183		169					
crude nuclei	43	x	90	78	140,389	25,780	1,536	334
crude mitochondria	35	15	90	13	131,848	5,750	1,460	416
heavy microsomes	12	4	5	5	2,484	3,251	486	770
light microsomes	15	4	6	5	7,136	13,086	1,285	2,676
final supernatant	42	29	18	78	6,576	168,131	369	2,154

- = no ruthenium red in homogenizing medium

+ = ruthenium red in homogenizing medium

x not determined due to ruthenium red interference

Part 3.23.3.2 The effect of inhibitors on calcium distribution in the mucosal cell loaded in vivo with calcium3.3.2.1 The effect of sodium fluoride on calcium distribution in the subcellular fraction of loaded mucosal cellsMETHOD

The duodenum mucosal cells of starved rachitic and vitamin D₃-replete chicks were loaded with 500 μ l $^{45}\text{Ca}^{2+}/^{40}\text{Ca}^{2+}$ solution (2 mg, 5 μ Ci) for 20 min as described 1.4.1 and 1.4.2. The loops were washed out internally with 5 ml ice-cold 0.25M-sucrose + 2mM-Mg²⁺ + 10⁻³M-NaF. The mucosal cells were isolated and homogenized in 0.25M-sucrose + 2mM-Mg²⁺ + 10⁻³M-NaF and fractionated as described in 1.5.1, 1.5.2, 1.5.3.1 and 1.5.3.2. Blood and bone samples were taken at the end of 20 min loading period and treated as described in 1.7.3.1 and 1.7.4.1 respectively.

RESULTS

The bone and blood $^{45}\text{Ca}^{2+}$ content of vitamin D₃-replete chick was greater by 4.5 fold and 2.5 fold respectively than those of the rachitic chicks. Thus at least 2.5 times as much $^{45}\text{Ca}^{2+}$ was passing through the vitamin D₃ chick mucosal cells. The rachitic mitochondria and main residue have retained more of the $^{45}\text{Ca}^{2+}$ than vitamin D₃-replete chicks (Table 25). A large proportion of the Ca^{2+} was associated with the mitochondrial fraction and very little in the supernatant. The distribution of Ca^{2+} within the organelles was similar for both types of chick and similar to that observed in 2.2.1 in the absence of inhibitors.

TABLE 25

The effect of sodium fluoride on the subcellular distribution of calcium within the mucosal cells of the duodenum

Duodenum mucosal cells of rachitic and vitamin D₃-replete chicks were loaded with 500 μ l $^{45}\text{Ca}^{2+}/^{40}\text{Ca}^{2+}$ solution (2 mg, 5 μCi) for 20 min as described in 1.4.1 and 1.4.2. The loops were washed out with 5 ml 0.25M-sucrose + 2mM-Mg²⁺ + 10⁻³M-NaF. The cells were isolated and homogenized in the above medium as described in 1.5.1 and 1.5.2. The fractionation was done as described in 1.5.3.1. Blood and bone samples were taken from each chick at the end of the 20 min period and were treated as described in 1.7.3.1 and 1.7.4.1 respectively. The results are average for 2 duodena expressed as per duodenum.

Fraction	Type of chick	protein mg	$^{40}\text{Ca}^{2+}$ μg	C.P.M.	C.P.M./ $^{40}\text{Ca}^{2+}$ μg
main residue	Vitamin D ₃ -treated	39	37	76,026	2,048
	Rachitic	69	65	180,929	2,804
crude mitochondria	Vitamin D ₃ -treated	53	340	587,053	1,726
	Rachitic	54	220	711,981	3,207
microsomes	Vitamin D ₃ -treated	31	13	19,215	1,459
	Rachitic	28	18	32,711	1,859
final supernatant	Vitamin D ₃ -treated	50	5	279	56
	Rachitic	49	6	48	8

^{45}Ca present in the blood after 20 min period were as follows: vitamin D₃-replete chicks 6,960 C.P.M./ml plasma and rachitic chicks 2,500 C.P.M./ml plasma and the tibia had accumulated: vitamin D₃-replete chicks 45,539 C.P.M./ashed tibia and rachitic chicks 9,454 C.P.M./ashed tibia.

3.3.2.2 The effect of dicoumarol on the subcellular distribution of calcium within the mucosal cells of the duodenum

METHOD

Duodenal loops of starved rachitic and vitamin D₃-replete chicks were loaded with 500 μ l $^{45}\text{Ca}^{2+}/^{40}\text{Ca}^{2+}$ solution (2 mg, 5 μ Ci) for 20 min as described in 1.4.1 and 1.4.2. The loops were washed out with 0.25M-sucrose with O_2 without 10^{-4} M-dicoumarol. The mucosal cells were isolated and homogenized in 0.25M-sucrose + 2mM-Mg²⁺ with O_2 without 10^{-4} M-dicoumarol as described in 1.5.1 and 1.5.2, and fractionated in media with O_2 without dicoumarol as described in 1.5.3.1. Blood and bone samples were taken and treated as described in 1.7.3.1 and 1.7.4.1 respectively.

RESULTS

The effect of this inhibitor on the loaded cells is shown in Table 26. It was shown in 3.2.1.2 that $^{45}\text{Ca}^{2+}$ loaded in vivo into mitochondria was released when these mitochondria were placed into media containing dicoumarol. Dicoumarol was also shown to prevent uptake of $^{45}\text{Ca}^{2+}$ by mitochondria and the homogenate at 0°C (cf. tables 13 and 14). It appears that this inhibitor has caused release of $^{45}\text{Ca}^{2+}$ and possibly of endogenous $^{40}\text{Ca}^{2+}$ from the nuclear and mitochondrial fractions as shown by the specific activity of the $^{45}\text{Ca}^{2+}$ in the supernatant (Table 26). There has been no discrimination by the inhibitor for either of the types of preparations (vitamin D₃-replete or rachitic).

TABLE 26

The distribution of calcium in duodenum mucosal cells organelles in the presence and absence of dicoumarol at 0°C

4 duodenal loops of rachitic and vitamin D₃-replete chicks were loaded with 500 μ l $^{45}\text{Ca}^{2+}/^{40}\text{Ca}^{2+}$ solution (2 mg, 5 μ Ci) for 20 min. The loops were washed out with 0.25M-sucrose with or without 10^{-4} M-dicoumarol at 0°C. The mucosal cells were isolated as described in 1.5.1 and homogenized in 0.25M-sucrose + 2mM-Mg²⁺ with or without 10^{-4} M-dicoumarol as described in 1.5.2 and fractionated in media containing dicoumarol as described in 1.5.3.1. Blood and bone samples were taken at end 20 min loading period and were treated as described in 1.7.3.1 and 1.7.4.1 respectively. The results are the average of 3 separate experiments (4 chicks per group) expressed as per duodenum. The bone and blood results are the average for 2 chicks in each group.

Table 26

Fraction	Type of chick	protein mg		$^{40}\text{Ca}^{2+}$ μg		C.P.M.		C.P.M./ $^{40}\text{Ca}^{2+}$ μg	
		-*	+*	-	+	-	+	-	+
main residue	Vitamin D ₃ -treated	68	53	145	84	417,368	331,659	2,878	3,948
	Rachitic	60	74	210	135	686,012	378,046	3,226	2,800
crude mitochondria	Vitamin D ₃ -treated	70	58	128	65	348,431	202,697	2,722	3,118
	Rachitic	47	62	219	67	622,286	179,261	2,841	2,675
Microsomes	Vitamin D ₃ -treated	25	23	10	32	5,388	13,838	538	432
	Rachitic	20	20	11	10	6,599	12,334	599	1,233
final supernatant	Vitamin D ₃ -treated	54	57	9	119	3,135	310,918	348	2,617
	Rachitic	56	48	11	136	3,161	352,923	287	2,595

* - = Fractionation medium without dicoumarol

* + = Fractionation medium with dicoumarol

$^{45}\text{Ca}^{2+}$ present in blood plasma after 20 min; vitamin D₃-replete chick 7,500 C.P.M./ml and rachitic chick 2,550 C.P.M./ml.

$^{45}\text{Ca}^{2+}$ accumulated by tibia in 20 min; vitamin D₃-replete chick 50,373 C.P.M./ashed tibia and rachitic chick 12,277 C.P.M./ashed tibia.

3.3.2.3 The effect of antimycin plus warfarin on the subcellular distribution of calcium in mucosal cells of the duodenum

METHOD

Duodenal loops of starved rachitic chicks were loaded with 500 μ l $^{45}\text{Ca}^{2+}/^{40}\text{Ca}^{2+}$ solution (2 mg, 5 μ Ci) for 20 min as described in 1.4.1 and 1.4.2. The mucosal cells were isolated and homogenized in 0.25M-sucrose + 2mM-Mg $^{2+}$ with or without inhibitors (10^{-5} M-antimycin + 10^{-4} M-warfarin) as described in 1.5.1 and 1.5.2. The homogenate was fractionated in media with or without the inhibitors (1.5.3.1 and 1.5.3.2).

RESULTS

The results in Table 27 show that antimycin plus warfarin present during homogenation have no effect on the distribution of Ca^{2+} in the organelles. The mitochondrial fraction contained most of the Ca^{2+} and the highest specific activity of $^{45}\text{Ca}^{2+}$. Thus antimycin plus warfarin have not caused release of Ca^{2+} from these organelles so isolated. The final supernatant contained about 2% of the total Ca^{2+} of the cell.

TABLE 27

The effect of antimycin and warfarin on the cellular distribution of calcium in duodenum mucosal cell at 0°C

Duodenal loops of 3 starved rachitic chicks were loaded with 500 μ l $^{45}\text{Ca}^{2+}/^{40}\text{Ca}^{2+}$ solution (2 mg, 5 μCi) for 20 min. The mucosal cells were isolated (1.5.1) and homogenized in 0.25M-sucrose + 2mM- Mg^{2+} , with or without 10^{-4} M-antimycin + 10^{-4} M-warfarin (1.5.2). The homogenate was fractionated in media with or without the inhibitors as described in 1.5.3.1 and 1.5.3.2. The results are the average for 3 experiments expressed as per duodenum.

Fraction	protein mg		$^{40}\text{Ca}^{2+}$ μg		C.P.M.		C.P.M./ $^{40}\text{Ca}^{2+}$ μg	
	-*	+*	-	+	-	+	-	+
original homogenate	168	244	501	506	1,380,563	1,754,530	2,788	3,404
crude nuclei	25	26	100	52	230,686	113,751	2,214	2,276
crude mitochondria	57	55	190	217	495,450	625,807	2,608	2,884
heavy microsomes	12	12	6	7	9,164	11,880	1,655	1,942
light microsomes	18	17	5	5	10,282	9,512	1,916	1,754
final supernatant	43	46	15	14	22,765	10,467	1,482	791

* - = homogenizing and fractionation media without antimycin plus warfarin

* + = homogenizing and fractionation media with antimycin plus warfarin

3.3.2.4 The effect of antimycin plus warfarin, administered to the live chick before removal of duodenal loop, on calcium distribution in the homogenate

METHOD

Since antimycin is said to prevent Ca^{2+} uptake by mitochondria under conditions of oxidative phosphorylation (Crofts and Chappell, 1965), an attempt was made to prevent redistribution of Ca^{2+} by giving the inhibitors to the chick just before removing the duodenal loop.

0.3 ml of propylene glycol containing 1.2 mg antimycin and 0.19 mg warfarin were injected intraperitoneally into chick 10 min after placing $^{45}\text{Ca}^{2+}$ into the duodenal loop (1.4.1 and 1.4.2). The loop was removed after a total of 15 min absorbing period and the cells isolated and homogenized in 0.25M-sucrose + 2mM- Mg^{2+} + 10^{-5}M -antimycin + 10^{-4}M -warfarin (1.5.1 and 1.5.2) and fractionated (1.5.3.1 and 1.5.3.2).

RESULTS

There appears to be very little effect of these inhibitors upon the Ca^{2+} distribution in the duodenal cells of the rachitic or vitamin D_3 -replete chicks. The final supernatants have a greater percent of $^{45}\text{Ca}^{2+}$ and $^{40}\text{Ca}^{2+}$ - 6% and 4%, and 12% and 9% for vitamin D_3 -replete and rachitic chicks respectively - than normally obtained without inhibitors (Table 28). Thus antimycin plus warfarin may have interfered with the removal of $^{45}\text{Ca}^{2+}$ from the cell into the extracellular fluid, and this would account for the greater accumulation in the vitamin D-replete cells.

TABLE 28

The effect of administration of antimycin plus warfarin to the live chick on subcellular distribution of calcium

Duodenal loops of rachitic and vitamin D₃-replete chicks were loaded with 500 μ l $^{45}\text{Ca}^{2+}/^{40}\text{Ca}^{2+}$ solution (2 mg, 5 μ Ci) for 15 min. Ten minutes after $^{45}\text{Ca}^{2+}$ solution addition, 0.3 ml propylene glycol containing 1.2 mg antimycin and 0.19 mg warfarin were injected intraperitoneally. The mucosal cells were isolated as described in 1.5.1 after washing out the loop with 5 ml 0.25M-sucrose + 2mM-Mg²⁺ + 10⁻⁵M-antimycin + 10⁻⁴M-warfarin. The cells were homogenized in this medium as described in 1.5.2 and fractionated as described in 1.5.3.1 and 1.5.3.2. A bone sample was taken after 15 min loading period and treated as described in 1.7.4.2. The results are the average of 2 duodena expressed as per duodenum.

Table 28

Fraction	Type of chick	protein mg	$^{40}\text{Ca}^{2+}$ μg	C.P.M.	C.P.M./ $^{40}\text{Ca}^{2+}$ μg
nuclei	Vitamin D ₃ - treated	41	64	108,421	1,694
	Rachitic	36	60	87,074	1,446
top mitochondria	Vitamin D ₃ - treated	24	18	27,325	1,489
	Rachitic	16	12	16,218	1,402
middle mitochondria	Vitamin D ₃ - treated	23	83	161,521	1,947
	Rachitic	22	86	146,035	1,705
heavy mitochondria	Vitamin D ₃ - treated	10	85	124,898	1,470
	Rachitic	7	45	58,641	1,290
heavy microsomes	Vitamin D ₃ - treated	26	12	17,544	1,415
	Rachitic	28	11	14,680	1,385
light microsomes	Vitamin D ₃ - treated	22	12	13,853	1,136
	Rachitic	33	10	10,411	1,041
final supernatant	Vitamin D ₃ - treated	66	39	29,162	753
	Rachitic	69	23	16,441	709

^{45}Ca accumulated by tibia in 15 min; vitamin D₃ chick 6,656 C.P.M./
whole tibia and rachitic chick 2,348 C.P.M./whole tibia.

3.3.2.5 The effect of EGTA on the subcellular calcium distribution in mucosal cell of duodenum

METHOD

3.3.2.5.1 The duodenal loops of starved rachitic chicks were loaded as described in 1.4.1 and 1.4.2. The loop was washed out with 5 ml 0.154M-NaCl. The cells were isolated (1.5.1) and placed in preweighed ice-cold beaker, weighed, mixed gently and divided into two thirds and one third portions. The two thirds portion of the cells was homogenized in 0.25M-sucrose + 50mM-Tris-Tricine pH 7.1 + 2mM-Mg²⁺. The homogenate was divided into two equal portions. To one half was added 5 x 10⁻²M-Tris EGTA pH 7.1 to make 1mM, while to the other half nothing was added. The one third portion of the cells was homogenized in 0.25M-sucrose + 50mM-Tris Tricine pH 7.1 + 2mM-Mg²⁺ + 1mM-EGTA after standing for 2 min in this media. The homogenates were fractionated (1.5.3.1 and 1.5.3.2).

3.3.2.5.2 The duodenal loops of starved rachitic and vitamin D₃-replete were loaded as described in 1.4.1 and 1.4.2. The loops were washed out with 5 ml 0.25M-sucrose + 2mM-Mg²⁺ + 1mM-Tris EGTA pH 7.1 and washed externally with 0.154M-NaCl. The mucosal cells were isolated (1.5.1) and homogenized in 0.25M-sucrose + 2mM-Mg²⁺ + 1mM-Tris EGTA (1.5.2) and the homogenate fractionated (1.5.3.1 and 1.5.3.2). A bone sample was taken from each chick and treated as described in 1.7.4.2.

RESULTS

3.3.2.5.1 Table 29 shows that EGTA has caused Ca²⁺ to be released from nuclear and mitochondrial fractions after addition to the

homogenized cells. The effect of EGTA on the subcellular distribution is less dramatic than that shown by dicoumarol (cf. Table 21). As shown in 3.2, EGTA inhibited Ca^{2+} uptake by mitochondria but did not cause release at 0°C . Thus homogenation in the presence of EGTA possibly prevented $^{45}\text{Ca}^{2+}$ from being taken up by the mitochondrial and nuclear fractions. But it is not evident in this experiment from which fraction the $^{45}\text{Ca}^{2+}$ has either been released or its uptake prevented, although the specific activity of $^{45}\text{Ca}^{2+}$ was slightly decreased in both nuclear and mitochondrial fractions. However not all the $^{45}\text{Ca}^{2+}$ present in the supernatant could be accounted for by release from mitochondria fraction.

3.3.2.5.2 In Table 30 a comparison is shown of $^{45}\text{Ca}^{2+}$ distribution in vitamin D_3 -replete and rachitic mucosal cells of the duodenum under the influence of EGTA in the washing out fluid and homogenizing medium. Evidently EGTA has either prevented $^{45}\text{Ca}^{2+}$ uptake from the cytoplasm, or has caused release from the nuclear and mitochondrial fractions as in the previous experiment (Table 29), or both mechanisms may have been operating, leading to over half of the $^{45}\text{Ca}^{2+}$ remaining in the soluble phase. The cellular contents are similar even though there is 3 times as much Ca^{2+} passing through the vitamin D_3 -treated cells.

TABLE 29

The effect of EGTA on the subcellular calcium distribution of duodenum mucosal cells at 0°C

6 duodenal loops of starved rachitic chicks were loaded with $500 \mu\text{l } ^{45}\text{Ca}^{2+}/^{40}\text{Ca}^{2+}$ solution (2 mg, 5 μCi) for 20 min. The mucosal cells were isolated after washing the loop externally and internally 0.15M-NaCl (1.5.1). The cells were placed in a preweighed ice-cold beaker, weighed, mixed gently and divided into 2 portions: - one of two thirds and the other one third of cells. The two third portion was homogenized in 0.25M-sucrose + 50mM-Tris Tricine pH 7.1 + 2mM-Mg²⁺ at 0°C and subsequently the homogenate was divided equally. To half of this homogenate was added $5 \times 10^{-3}\text{M}$ -EGTA to make 10^{-3}M , and to the other half nothing was added. The one third portion of the mucosal cells was homogenized in 0.25M-sucrose + 50mM-Tris Tricine + 2mM-Mg²⁺ + 10^{-3}M -EGTA after standing for about 2 min in this media. The homogenates were centrifuged at 600g for 10 min. The pellet rehomogenized and added to the supernatant. A sample of the original homogenate was taken. The homogenate was layered over 0.34M-sucrose + 50mM-Tris Tricine + 10^{-3}M -EGTA and centrifuged at 1000g for 10 min. This was fractionated as described in 1.5.3.1 and 1.5.3.2. The results are expressed as per duodenum.

Table 29

Fraction	protein mg			$^{40}\text{Ca}^{2+}$ μg			C.P.M.			C.P.M./ $^{40}\text{Ca}^{2+}$ μg		
	-	E1	E2	-	E1	E2	-	E1	E2	-	E1	E2
original homogenate	194	189	188	367	386	412	1,113,212	1,117,088	1,391,051	3,033	2,894	3,376
nuclei	26	28	23	12	16	11	25,680	13,853	15,038	2,140	874	1,432
mitochondria	73	78	82	273	300	326	789,113	664,423	724,452	2,891	2,215	2,222
heavy microsomes	18	15	15	9	9	7	15,061	11,835	13,418	1,721	1,347	1,877
light microsomes	23	17	18	9	8	8	10,408	7,223	9,190	1,125	897	1,194
final supernatant	52	43	44	16	45	55	26,522	152,845	236,607	1,637	3,370	4,298

* E1 = EGTA added to homogenate after homogenation

* E2 = EGTA in homogenizing medium during homogenation

* — = nothing added or in homogenizing medium

TABLE 30

The effect of EGTA on the subcellular distribution of calcium in the mucosal cells of rachitic and vitamin D₃-replete chick duodena at 0°C

Duodenal loops of starved rachitic and vitamin D₃-replete chick were loaded with 500 μ l $^{45}\text{Ca}^{2+}/^{40}\text{Ca}^{2+}$ solution (2 mg, 5 μ Ci) for 20 min. The loops were washed out with 5 ml 0.25M-sucrose + 10^{-3} M-EGTA and washed externally with 0.154M-NaCl. The mucosal cells were isolated as described in 1.5.1, homogenized in 0.25M-sucrose + 2mM-Mg²⁺ with or without 1mM-EGTA as described in 1.5.2 and fractionated as described in 1.5.3.1 and 1.5.3.2. A bone sample was taken from each chick and treated as described in 1.7.4.2. The results are from 3 experiments with 3 birds per group expressed as per duodenum.

TABLE 30

Fraction	Type of chick	protein mg	$^{40}\text{Ca}^{2+}$ μg	C.P.M.	C.P.M./ $^{40}\text{Ca}^{2+}$ μg
crude nuclei	Vitamin D ₃ -treated	58	37	92,848	2,437
	Rachitic	56	35	94,898	2,480
crude mitochondria	Vitamin D ₃ -treated	39	76	183,421	2,509
	Rachitic	43	59	167,748	2,772
heavy microsomes	Vitamin D ₃ -treated	14	8	16,491	2,171
	Rachitic	13	7	14,999	2,219
light microsomes	Vitamin D ₃ -treated	18	8	15,700	2,035
	Rachitic	17	6	14,980	2,280
final supernatant	Vitamin D ₃ -treated	58	135	507,913	3,742
	Rachitic	59	182	730,705	4,018

$^{45}\text{Ca}^{2+}$ accumulated by tibia in 20 min; vitamin D₃ chick 28,168

C.P.M./whole tibia and rachitic chick 6,440 C.P.M./whole tibia.

3.3.2.6 The effect of ruthenium red on the subcellular calcium distribution in the mucosal cells at 0°C

METHOD

Duodenal loops of rachitic starved chicks were loaded with 500 μ l $^{45}\text{Ca}^{2+}/^{40}\text{Ca}^{2+}$ solution (20 μ g, 2 μ Ci) for 20 min as described in 1.4.1 and 1.4.2. The mucosal cells were isolated (1.5.1) and homogenized in 0.25M-sucrose + 50mM-Tris Tricine pH 7.1 + 2mM-Mg²⁺ + 1.4 mg/ml ruthenium red at 0°C (1.5.2). The homogenate was centrifuged at 78,000g for 30 min at 2°C.

In a similar experiment chicks were starved or fed but not loaded with $^{45}\text{Ca}^{2+}$ and the distribution of Ca^{2+} in the final supernatant was examined.

RESULTS

In Table 31A the effect of ruthenium red on the distribution of $^{45}\text{Ca}^{2+}$ in the homogenate is shown. The chicks were given only a small amount of $^{45}\text{Ca}^{2+}$ so as to minimise the soluble Ca^{2+} . In the presence of ruthenium red, a greater percent of $^{45}\text{Ca}^{2+}$ was found in the soluble fraction of the cell, in spite of the fact that a considerable amount of the cell protein has been precipitated by the ruthenium red. This is also evident in Table 31B where starved and fed non-loaded cells from rachitic duodena were isolated and homogenized in medium containing ruthenium red; here again about 15% remained in the cytoplasm.

TABLE 31A

The effect of ruthenium red on calcium distribution in calcium loaded mucosal cell at 0°C

Duodenal loops of 3 starved rachitic chicks were loaded with 500 μ l $^{45}\text{Ca}^{2+}/^{40}\text{Ca}^{2+}$ solution (20 μ g, 2 μ Ci) for 20 min (1.4.1 and 1.4.2). The mucosal cells were isolated and homogenized in 0.25M-sucrose + 50mM-Tris Tricine pH 7.1 + 2mM-Mg $^{2+}$ + 1.4 mg/ml ruthenium red at 0°C (1.5.1 and 1.5.2). The homogenate was centrifuged at 78,000g for 30 min. The residue was digested as described in 1.7.5. The results are expressed as per duodenum.

Fraction	protein mg	$^{40}\text{Ca}^{2+}$ μ g	C.P.M.
residue	-	80	320,977
supernatant	50	12	51,983
% in supernatant	-	13	14

- not determined due to interference from ruthenium red

TABLE 31B

The effect of ruthenium red on calcium distribution in mucosal cell (non-loaded) at 0°C

Duodenum mucosal cells were isolated from 3 fed and 3 starved rachitic chicks (1.5.1). The cells were homogenized in 0.25M-sucrose + 2mM-Mg²⁺ + 50mM-Tris Tricine + 1.4 mg/ml ruthenium red at 0°C (1.5.2). The homogenate was centrifuged at 78,000g for 30 min. The residue was digested as described in 1.7.5. The results are expressed as per duodenum.

Fraction	condition	protein mg	⁴⁰ Ca ²⁺ μg
residue	starved	-	130
	fed	-	109
supernatant	starved	48	40
	fed	44	20
% in	starved	-	24
supernatant	fed	-	15

- not determined due to interference from ruthenium red

3.3.2.7 The effect of intraperitoneal administration of rotenone plus warfarin and ruthenium red in the homogenizing medium on the subcellular distribution of calcium in the mucosal cell

METHOD

In this experiment an attempt was made to trap the $^{45}\text{Ca}^{2+}$ being absorbed by giving these inhibitors to the live chick before removing the duodenum and then homogenizing with ruthenium red as in the previous experiment. Antimycin plus warfarin, as reported above, would have been the inhibitor of choice, but antimycin became unavailable in Australia at this time, so rotenone plus warfarin was substituted. Antimycin and rotenone have similar effect on Ca^{2+} uptake by mitochondria (Carafoli et al, 1966).

Preliminary experiments were done to find out the maximum amount of rotenone that could be used in these experiments by injecting a mixture of rotenone plus warfarin dissolved in propylene glycol intraperitoneally and studying the survival over a period of 1 to 16 h. A suitable sublethal dose was 0.18 ml propylene glycol containing 0.72 mg rotenone plus 1.08 mg warfarin per 180g chick.

The duodenal loops of starved vitamin D_3 -replete chicks were loaded with 500 μl $^{45}\text{Ca}^{2+}/^{40}\text{Ca}^{2+}$ solution (2 mg, 5 μCi) for 20 min (1.4.1 and 1.4.2). The chicks were injected intraperitoneally with 0.18 ml propylene glycol containing 0.72 mg rotenone + 1.08 mg warfarin, 10 min before and also 15 min after loading the loop. At the end of the 20 min absorption period the loop was removed, washed externally and internally. The mucosal cells were isolated (1.5.1). The cells were homogenized in 0.25M-sucrose + 50mM-Tris Tricine pH 7.1 + 2mM- Mg^{2+} + 1.4 mg/ml ruthenium red at 0°C . The homogenate was

centrifuged at 78,000g for 30 min at 2°C. A bone sample was taken at the end of the 20 min period and treated as described in 1.7.4.2.

RESULTS

The administration of rotenone plus warfarin 10 min before and 15 min after $^{45}\text{Ca}^{2+}$ loading of the duodenal loop has had no effect on Ca^{2+} translocation as there was no difference in $^{45}\text{Ca}^{2+}$ content of bone as compared to the control. However rotenone plus warfarin with ruthenium red has increased the cytoplasmic Ca^{2+} content to 14% as compared with the control of 9%. Ruthenium red alone as shown in 3.3.2.6 increased the $^{45}\text{Ca}^{2+}$ in the final supernatant, and in this experiment $^{45}\text{Ca}^{2+}$ in the control group supernatant was 9% of the total $^{45}\text{Ca}^{2+}$ (Table 32).

TABLE 32

The effect of rotenone plus warfarin given to the live chick on the calcium distribution in mucosal cell homogenate of the duodenum

The duodenal loops of starved vitamin D₃-replete chicks were loaded with 500 μ l $^{45}\text{Ca}^{2+}/^{40}\text{Ca}^{2+}$ solution (2 mg, 5 μ Ci) for 20 min (1.4.1 and 1.4.2). The chicks were injected intraperitoneally with 0.18 ml propylene glycol containing 0.72 mg rotenone + 1.08 mg warfarin, 10 min before and also 15 min after loading the loop. At the end of the 20 min absorption period the loop was removed, washed externally and internally. The mucosal cells were isolated (1.5.1). The cells were homogenized in 0.25M-sucrose + 50mM-Tris Tricine pH 7.1 + 2mM-Mg²⁺ + 1.4 mg/ml ruthenium red at 0°C. The homogenate was centrifuged at 78,000g for 30 min at 2°C. A bone sample was taken at the end of the 20 min period and was treated as described in 1.7.4.2. The results are the average of 3 duodena expressed as per duodenum.

Fraction	protein mg		$^{40}\text{Ca}^{2+}$ ug		C.P.M.		C.P.M./ $^{40}\text{Ca}^{2+}$ ug	
	-*	+*	-	+	-	+	-	+
residue	-	-	181	155	1,036,649	929,073	6,196	6,118
supernatant	45	36	19	25	106,421	161,275	5,711	6,486
% in supernatant			9	14	9	15	-	-

The tibia had accumulated in 20 min period; vitamin D₃-replete chick (control) 26,861^x C.P.M./whole tibia and vitamin D₃-replete chick (rotenone + warfarin) 32,486^x C.P.M./whole tibia.

x not statistically significant

* - = chicks were not injected with rotenone + warfarin

* + = chicks were injected with rotenone + warfarin

PART 43.4 The effect of certain inhibitors on calcium distribution in microsomal cells at 22°C or above

In most of the work on cell fractionation done in this thesis or by the majority of research workers, the cells were cooled in ice-cold media. There seems a danger that even before homogenation a redistribution of Ca^{2+} could take place. Therefore in this section attempts were made to keep the cells and homogenate above 20°C during all manipulations.

For work at 20°C or above careful selection of inhibitors of Ca^{2+} accumulation had to be made. Some inhibitors eg EGTA caused release of Ca^{2+} from organelles at this temperature. Inhibitors that have little effect on Ca^{2+} accumulation by mitochondria at 0°C eg. antimycin and rotenone, are known to inhibit uptake at 30°C (Carafoli et al 1966). Ruthenium red inhibits Ca^{2+} uptake at 0°C and 30°C and has little effect on causing release from organelles and would therefore appear to be the most useful inhibitor for studying Ca^{2+} present in the cytoplasm. In this section ruthenium red at 22°C was used to estimate the Ca^{2+} present in the cytoplasm during the absorption of Ca^{2+} from the duodenal loops.

Advantage was taken of the rapid separation possible in a zonal rotor to compare the distribution of $^{45}\text{Ca}^{2+}$ present in the homogenates prepared at 0°C and 28°C.

Part 4.13.4.1 The effect of the temperature of homogenation on calcium distribution in the mucosal cellMETHOD

Rachitic starved chicks were loaded with $^{45}\text{Ca}^{2+}$ as described in 1.4.1 and 1.4.2. The isolated mucosal cells were homogenized at 0°C or 22°C as described in 1.5.1 and 1.5.2. The homogenate prepared at 0°C was divided into 2 equal portions. One portion was left at room temperature (22°C) for $1\frac{1}{4}$ h. The homogenates prepared at 22°C and 0°C were immediately centrifuged at 78,000g for 30 min at the appropriate temperature. The homogenate, allowed to attain room temperature over a period of $1\frac{1}{4}$ h from 0°C , was centrifuged at 78,000g for 30 min at 22°C . Enzyme assays were done on the supernatant and homogenates (1.9). Protein, $^{40}\text{Ca}^{2+}$ and $^{45}\text{Ca}^{2+}$ were determined for each fraction (1.8).

RESULTS

The supernatant of the homogenate prepared at 0°C and 22°C contained 6% and 18% of $^{40}\text{Ca}^{2+}$ and 1% and 13% $^{45}\text{Ca}^{2+}$. However the supernatant of the homogenate prepared at 0°C that was allowed to attain room temperature contained 36% $^{40}\text{Ca}^{2+}$ and 31% $^{45}\text{Ca}^{2+}$ (Table 33).

The enzyme assays done for malic dehydrogenase, succinic dehydrogenase and acid phosphatase showed that the content of malic dehydrogenase in the supernatant increased from 10% at 0°C to 18% at 22°C , while the supernatant of the homogenate brought to 22°C from 0°C contained 22%. The other 2 enzymes showed very little rise in activity in the supernatant indicating that the organelles were still virtually intact after the 3 treatments (Table 33). Beaufay, Bendall,

Baudhuin, and De Duve (1959) showed that 11% of malic dehydrogenase was present in the supernatant of a homogenate prepared at 0°C of rat liver.

TABLE 33

The effect of temperature of homogenation on calcium distribution in mucosal cell

Six duodenal loops of rachitic starved chicks were loaded with 500 μ l $^{45}\text{Ca}^{2+}/^{40}\text{Ca}^{2+}$ solution (2 mg, 5 μ Ci) for 20 min (1.4.1 and 1.4.2). Mucosal cells from 4 duodena were homogenized at 0°C in 0.25M-sucrose + 50mM-Tris Tricine pH 7.1 + 2mM-Mg²⁺ (1.5.1). One half of this homogenate was centrifuged immediately at 78,000g for 30 min at 0°C. The second half was allowed to come to 22°C over 1½ h. This homogenate was centrifuged at 22°C at 78,000g for 30 min. The other 2 duodenal loops mucosal cells were scraped, homogenized in 0.25M-sucrose + 2mM-Mg²⁺ + 50mM-Tris Tricine pH 7.1 and centrifuged as above at 22°C. Enzyme assays of malic dehydrogenase, succinic dehydrogenase and acid phosphatase were made as described in 1.9. The results are expressed as per duodenum.

TABLE 33

Temperatures of homogenized °C centrifuged °C	Protein mg			⁴⁰ Ca ²⁺ pg			C.P.M.			malic dehydrogenase			succinic dehydrogenase			acid phosphatase		
	0	0	22	0	0	22	0	0	22	0	0	22	0	0	22	0	0	22
	0	22	22	0	22	22	0	22	22	0	22	22	0	22	22	0	22	22
original homogenate	238	190	209	229	201	136	724,125	701,233	480,196	1.055	0.975	0.923	0.57	0.54	0.49	1.29	1.20	0.91
residue	158	130	154	208	125	108	712,105	482,331	413,608	-	-	-	-	-	-	-	-	-
supernatant	71	55	54	14	70	24	10,111	212,213	61,851	0.100	0.213	0.176	0.00	0.009	0.014	0.020	0.030	0.037
% in supernatant	31	30	26	6	36	18	1	31	13	10	22	18	0	2	3	1	2	3

Part 4.23.4.2 A comparison of the effect of inhibitors at 0°C and 30°C on calcium distribution in the homogenateMETHOD

Mucosal cells from $^{45}\text{Ca}^{2+}$ loaded duodena of starved rachitic chicks were isolated and placed into ice-cold beaker. The cells were equally proportioned by placing into 10 ml 0.25M-sucrose + 2mM-Mg $^{2+}$ with and without inhibitor at 0°C or 30°C. These cells were incubated in the medium for 5 min before homogenation. The homogenates were centrifuged at 78,000g for 30 min at the appropriate temperature.

RESULTS

Increasing the temperature of cells from 0°C to 30°C caused a release of $^{45}\text{Ca}^{2+}$ into the 78,000g supernatant (Table 34). There was very little effect of the inhibitors at 0°C except for EGTA which released 15% of the total ^{45}Ca . At 30°C, however, antimycin plus warfarin and EGTA both caused release to the supernatant of 35% and 42% respectively of the total ^{45}Ca . (Table 34).

TABLE 34

A comparison of the effect of inhibitors on the calcium distribution in the homogenate at 0°C and 30°C

Mucosal cells from $^{45}\text{Ca}^{2+}$ loaded duodenum of starved rachitic chicks were isolated (1.4.1, 1.4.2, 1.5.1) and placed into an ice-cold beaker. The cells were divided into portions, placed into 10 ml 0.25M-sucrose + 2mM-Mg $^{2+}$ with or without inhibitor at 0°C or 30°C, and incubated for 5 min in this medium. These mixtures were homogenized and then centrifuged at 78,000g for 30 min at the appropriate temperature. The results are expressed as approximately per duodenum.

Fraction	inhibitor	C.P.M.							
		none		antimycin (10^{-5}M) + warfarin (10^{-4}M)		rotenone (10^{-5}M)		EGTA (10^{-3}M)	
	temperature	0°C	30°C	0°C	30°C	0°C	30°C	0°C	30°C
residue		315,976	230,883	461,638	351,410	455,214	372,271	599,710	200,840
supernatant		3,080	27,381	21,756	185,834	4,313	39,653	102,990	147,096
% in supernatant		1	9	5	35	1	10	15	42

Part 4.33.4.3. The effect of rotenone plus warfarin and ruthenium red on calcium uptake by mucosal cells homogenate at 22°CMETHOD

Mucosal cells isolated from starved chick duodena were homogenized in 0.25M-sucrose + 2mM-Mg²⁺ + 50mM-Tris Tricine pH 7.2 at 0°C and 22°C (1.5.1 and 1.5.2). 5 ml of the homogenate was added to 5 ml 0.25M-sucrose + 50mM-Tris Tricine pH 7.2 with and without inhibitors and kept at 22°C or 0°C for 5 or 10 min before adding 200-250 µl ⁴⁵Ca²⁺/⁴⁰Ca²⁺ solution (200 µg/ml, 1 µCi). The mixtures were centrifuged at 78,000g for 30 min. ⁴⁰Ca²⁺, ⁴⁵Ca²⁺ and protein were estimated.

RESULTS

The cell homogenate at 0°C was shown to take up 98% of the added ⁴⁵Ca²⁺ (Table 35). However at 22°C the uptake was only 62% (Table 36). The amount of Ca²⁺ taken up from the homogenate at 22°C in the presence of ruthenium red was reduced to 44%. In the presence of rotenone plus warfarin alone the reduction was to 55%. When ruthenium red was also present rotenone plus warfarin reduced the uptake to 51%. It appears that there is only a small additive effect of ruthenium red plus rotenone plus warfarin on Ca²⁺ uptake.

TABLE 35

The uptake of calcium by the mucosal cell homogenate at 0°C

The mucosal cells from 3 duodenal loops of starved rachitic chicks were isolated and homogenized in 0.25M-sucrose + 2mM-Mg²⁺ + 50mM-Tris Tricine pH 7.1 as described in 1.5.1 and 1.5.2. 5 ml of the homogenate was preincubated at 0°C for 5 min with 5 ml 0.25M-sucrose + 50mM-Tris Tricine before adding 250 μ l ⁴⁵Ca²⁺ / ⁴⁰Ca²⁺ solution (50 μ g, 0.25 μ Ci). The homogenate was centrifuged at 78,000g for 30 min. The results are expressed as per duodenum as an average of 3 determinations. These results are control values at 0°C for comparison with Table 36.

Fraction	protein mg	⁴⁰ Ca ²⁺ μ g	C.P.M.
residue	126	284	74,947
supernatant	68	48	1,757
% in supernatant	35	14	2
% taken up	-	86	98

TABLE 36

The effect of rotenone plus warfarin and ruthenium red on calcium uptake by the cell homogenate at 22°C

Mucosal cells were isolated from duodena of 4 starved rachitic chicks and homogenized in 0.25M-sucrose + 2mM-Mg²⁺ + 50mM-Tris Tricine pH 7.2 at 22°C (1.5.1 and 1.5.2). 5 ml of the homogenate was added to 5 ml 0.25M-sucrose + 50mM-Tris Tricine Ph 7.2 with or without inhibitors and incubated at 22°C for 10 min before adding 200 μ l ⁴⁵Ca²⁺/⁴⁰Ca²⁺ solution (40 μ g, 0.2 μ Ci). The mixtures were centrifuged at 78,000g for 30 min. The results are expressed as per duodenum of a single experiment.

Fraction	inhibitor treatment		Protein mg	⁴⁰ Ca ²⁺ μ g	C.P.M.
	ruthenium red (1.4 mg/ml)	rotenone (10 ⁻⁴ M) + warfarin (2 x 10 ⁻⁴ M)			
residue	-	-	149	202	35,200
	+	-		151	22,851
	-	+	159	146	26,696
	+	+		166	22,824
supernatant	-	-	62	69	21,665
	+	-	49	127	28,810
	-	+	57	115	22,143
	+	+	53	126	21,854
			% of total		
in supernatant			⁴⁰ Ca ²⁺		⁴⁵ Ca ²⁺
	-	-	26		38
	+	-	46		56
	-	+	44		45
uptake of Ca ²⁺	+	+	43		49
	-	-	74		62
	+	-	54		44
	-	+	56		55
	+	+	57		51

Part 4.4

3.4.4 The effect of rotenone plus warfarin administered to live chicks on calcium distribution in the mucosal cell of duodenum after homogenation at 22°C in the presence of ruthenium red

METHOD

Five groups of starved rachitic and vitamin D₃-replete chicks were treated as follows:

GROUP 1: Mucosal cells of the duodenum were isolated and homogenized in 0.25M-sucrose + 2mM-Mg²⁺ + 1.4 mg/ml ruthenium red at 22°C (1.5.1 and 1.5.2).

GROUP 2: Thirty minutes before isolating the mucosal cells of the duodenum 0.18 ml propylene glycol containing 0.72 mg rotenone and 1.08 mg warfarin were administered intraperitoneally. The cells were isolated, homogenized and treated as above.

GROUP 3: 500 µl ⁴⁵Ca²⁺/⁴⁰Ca²⁺ solution (2 mg, 5 µCi) were placed in the duodenal loop for 20 min before isolating the mucosal cells.

The cells were homogenized and treated as above.

GROUP 4: 500 µl ⁴⁵Ca²⁺/⁴⁰Ca²⁺ solution (2 mg, 5 µCi) were placed in the duodenal loop for 20 min. Five min before removing the loop 0.18 ml propylene glycol containing rotenone and warfarin (as above) were injected intraperitoneally. The cells were isolated, treated and homogenized as above.

GROUP 5: Thirty minutes before removal of the duodenum 0.18 ml propylene glycol containing rotenone plus warfarin were injected intraperitoneally. Twenty minutes before removal of the loop, 500 µl ⁴⁵Ca²⁺/⁴⁰Ca²⁺ solution (2 mg, 5 µCi) were placed in the duodenal loop. The mucosal cells were isolated, homogenized and treated as above.

The homogenates were centrifuged at 78,000g for 30 min at 22°C. The residue and supernatant were estimated for $^{40}\text{Ca}^{2+}$, $^{45}\text{Ca}^{2+}$ and protein.

Bone samples were taken from the chicks loaded with $^{45}\text{Ca}^{2+}$.

The results are expressed as per duodenum as the average for 3 duodena.

RESULTS

Table 37 shows that the endogenous Ca^{2+} distribution in the cell at 22°C was unaltered by the administration of rotenone plus warfarin to the chick. In the loaded or non-loaded cell the Ca^{2+} content of the supernatant was about the same at approximately 34%. Rotenone plus warfarin administration 30 min before the removal of the $^{45}\text{Ca}^{2+}$ loaded loop has caused an increase in $^{40}\text{Ca}^{2+}$ content of the supernatant from 27% to 43% for vitamin D_3 -treated chick and from 32% to 45% from rachitic chick and $^{45}\text{Ca}^{2+}$ content from 34% to 49% for vitamin D_3 -replete and from 32% to 44% for rachitic chick (Table 38). The bones counts show the usual vitamin D_3 effect and have not been decreased by the administration of the inhibitors (Table 38).

TABLE 37

The effect of rotenone plus warfarin on calcium distribution in mucosal cells of duodenum homogenized at 22°C temperature in the presence of ruthenium red

Group 1. Duodenum mucosal cells of 3 starved rachitic and 3 vitamin D₃-replete chicks were isolated, homogenized in 21 ml 0.25M-sucrose + 2mM-Mg²⁺ + 29.4 mg ruthenium red at 22°C.

Group 2. 30 min before removal of the duodenum from chicks, 0.18 ml propylene glycol containing 0.72 mg rotenone and 1.08 mg warfarin were injected intraperitoneally. After 30 min the loops were removed and homogenized as for group 1. The homogenates were centrifuged at 78,000g for 30 min at 22°C.

The results are the average of 3 duodena expressed as per duodenum.

Fraction	Type of chick	⁴⁰ Ca ²⁺	
		gp 1 μ g	gp 2
residue	Vitamin D ₃ -treated	95	113
	Rachitic	123	126
supernatant	Vitamin D ₃ -treated	53	37
	Rachitic	68	60
% Ca ²⁺ in supernatant	Vitamin D ₃ -treated	36	25
	Rachitic	36	32

TABLE 33

The effect of rotenone plus warfarin on calcium distribution in mucosal cells of duodenum homogenized at 22°C temperature in the presence of ruthenium red

GROUP 3. 500 μ l $^{45}\text{Ca}^{2+}/^{40}\text{Ca}^{2+}$ solution (2 mg, 5 μ Ci) were placed in duodenal loop for 20 min. The cells were isolated and homogenized as for group 1.

GROUP 4. 500 μ l $^{45}\text{Ca}^{2+}/^{40}\text{Ca}^{2+}$ solution (2 mg, 5 μ Ci) were placed in the duodenal loops for 20 min. 5 min before removal of the loop, 0.18 ml propylene glycol containing 0.72 mg rotenone and 1.08 mg warfarin were injected intraperitoneally. The cells were isolated and homogenized as for group 1.

GROUP 5. 30 min before removal of the duodenum chicks 0.18 ml propylene glycol containing 0.72 mg rotenone and 1.08 mg warfarin were injected intraperitoneally. 10 min after the injection, 500 μ l $^{45}\text{Ca}^{2+}/^{40}\text{Ca}^{2+}$ solution (2 mg, 5 μ Ci) were placed in the duodenal loop. The mucosal cells were isolated and homogenized as for group 1.

The homogenates were centrifuged at 78,000g for 30 min at 22°C. Bone samples were taken from chicks loaded with $^{45}\text{Ca}^{2+}$ after the 20 min loading period and were treated as described in 1.7.4.2. The results are the average of 3 duodena expressed per duodenum.

Table 38

Fraction	Type of chick	$^{40}\text{Ca}^{2+}$ μg			C.P.M.			C.P.M./ $^{40}\text{Ca}^{2+}$ μg		
		gp 3	gp 4	gp 5	gp 3	gp 4	gp 5	gp 3	gp 4	gp 5
residue	Vitamin D ₃ -treated	209	171	127	274,866	259,135	163,660	1,318	1,514	1,289
	Rachitic	226	303	214	361,204	565,207	347,894	1,597	1,866	1,623
supernatant	Vitamin D ₃ -treated	77	107	96	143,265	182,368	156,472	1,864	1,668	1,626
	Rachitic	107	225	173	166,454	421,080	275,150	1,559	1,669	1,586
		$^{40}\text{Ca}^{2+}$			% of total			$^{45}\text{Ca}^{2+}$		
supernatant	Vitamin D ₃ -treated	27	39	43	34	41	49			
	Rachitic	32	43	45	32	44	44			
bones C.P.M./ whole tibia	Vitamin D ₃ -treated				59,969 *	82,077 *	54,397 *			
	Rachitic				15,420 +	14,607 +	10,812 +			

* not significantly different from each other

+ not significantly different from each other

3.4.5 A comparison of calcium distribution in the call homogenate fractionated by zonal centrifuge

METHOD

Duodenal loops of starved rachitic chicks were loaded for 20 min with 500 μ l $^{45}\text{Ca}^{2+}/^{40}\text{Ca}^{2+}$ solution (2 mg, 7.5 μ Ci) as described in 1.4.1 and 1.4.2. The mucosal cells were isolated (1.5.1), placed in 0.25M-sucrose + 2mM- Mg^{2+} + 1mM- NaHCO_3 + 10^{-4} M chloroquine and homogenized for 15 s in a Silverson blender (Silverson Machine Ltd, Chesham, Bucks), and then for 15 s (3 strokes) in the Potter Elvehjem (loose fitting) at 0°C or 28°C. The homogenate was then filtered through 2 layers of cheese cloth. A sample was taken of the homogenate. The rest of the homogenate (35-40 ml) was applied to the Sorvall SZ-14 zonal rotor containing a linear gradient ranging from 0.5M- to 2.26M-sucrose. 200 ml of 2.62M-sucrose was used as the cushion.

The rotor was spun at 2,500 rev/min ($\approx 343g$) while the gradient and the homogenate were being applied (343g). The speed of the rotor was increased to 12,000 rev/min ($\approx 8,000g$) and spun for 30 min at 0°C or 28°C. The rotor was emptied by a peristaltic pump. Fractions of about 12 ml were collected (approx. 100 tubes). The contents of the tubes were mixed before samples were taken for $^{45}\text{Ca}^{2+}$, DNA and enzyme estimations (1.8 and 1.9). The sucrose concentration was measured by refractometers (Abbe-Bellingham and Stanley, Ltd. England, and Atago, Japan). The results are shown in graphical form.

RESULTS

The distribution of $^{45}\text{Ca}^{2+}$ as shown in figures 4A and 5A is associated with the succinic dehydrogenase, a marker for the mitochondria. There appears to be no correlation of $^{45}\text{Ca}^{2+}$ with DNA, or alkaline phosphatase, or 5' Nucleotidase (fig. 4A and B and 5A and B). The time chosen for centrifugation at 0°C did not allow isopycnic equilibrium to be reached and separation of the fraction containing acid phosphatase from the mitochondria was not obtained. Thus in fig. 5A there appears to be a correlation between $^{45}\text{Ca}^{2+}$ and acid phosphatase. However when the acid phosphatase fraction was separated from the mitochondrial fraction in the less viscous medium at 28°C , it can be seen that there is no correlation between $^{45}\text{Ca}^{2+}$ and acid phosphatase (fig. 4A). In fig. 4A the $^{45}\text{Ca}^{2+}$ associated with the succinic dehydrogenase fraction constituted 32% of the total $^{45}\text{Ca}^{2+}$, whereas after homogenation and fractionation at 0°C (fig. 5A), 72% was associated with the succinic dehydrogenase fraction.

FIGURE 4A

Zonal rotor fractionation at 28°C

4 rachitic chick duodena were loaded with 500 μ l $^{45}\text{Ca}^{2+}/^{40}\text{Ca}^{2+}$ solution (2 mg, 7.5 μ Ci) for 20 min (1.4.1 and 1.4.2). The mucosal cells were isolated (1.5.1) and homogenized with 0.25M-sucrose + 1mM- NaHCO_3 + 2mM- Mg^{2+} + 10^{-4} M-chloroquine at 28°C in Silverson Blender for 15s and then in Potter Elvehjem for 15s (3 strokes). 37.5 ml of the homogenate was applied to a linear sucrose gradient of 1000 ml from 0.5M to 2.26M and 200 ml 2.62M-sucrose as a cushion. The gradient contained 10^{-4} M-chloroquine + 1mM- NaHCO_3 . The Sorvall SZ-14 zonal rotor and the solutions were maintained at 28°C. The gradient and the homogenate were applied to rotor spinning at 2,500 rev/min (\cong 343g). The rotor speed was increased to 12,000 rev/min (\cong 8,000g) after the homogenate had been applied and was spun for 30 min. The contents of the rotor were emptied by peristaltic pump. Fractions of about 12 ml were collected. The contents of tubes were mixed before visual observation by phase contrast microscopy, $^{45}\text{Ca}^{2+}$ and DNA estimation, enzyme assays and sucrose concentration were determined (1.8 and 1.9).

- — — — ● sucrose gradient (S.G.) (left hand scale)
- O ————— O acid phosphatase (Ac.P.) (left hand scale)
- X - - - - X succinic dehydrogenase (S.D.) (left hand scale)
- ————— □ $^{45}\text{Ca}^{2+}$ (right hand scale)

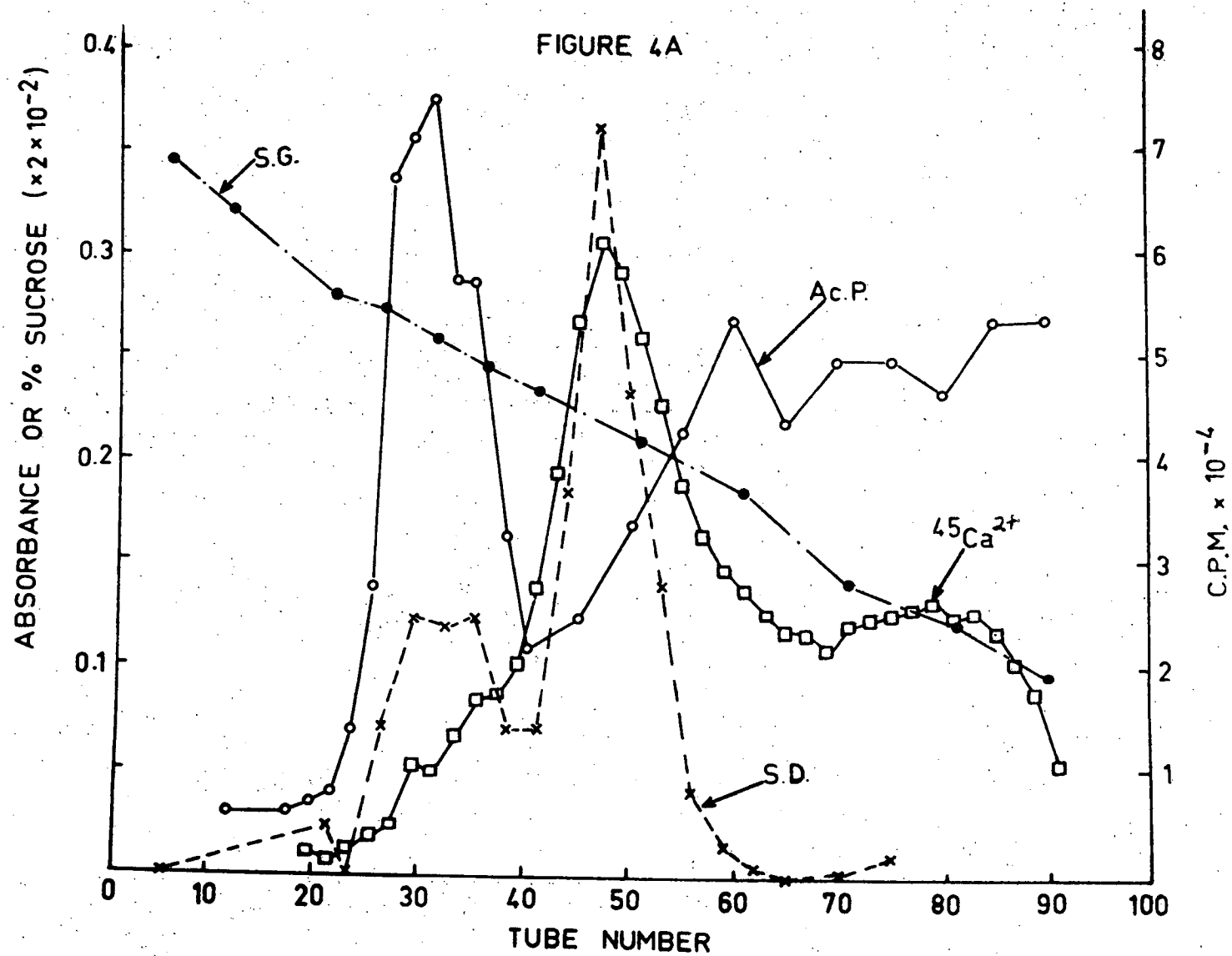


FIGURE 4B

Zonal rotor fractionation at 28°C

4 rachitic chick duodena were loaded with 500 μ l $^{45}\text{Ca}^{2+}/^{40}\text{Ca}^{2+}$ solution (2 mg, 7.5 μ Ci) for 20 min (1.4.1 and 1.4.2). The mucosal cells were isolated (1.5.1) and homogenized with 0.25M-sucrose + 1mM- NaHCO_3 + 2mM- Mg^{2+} + 10^{-4} M-chloroquine at 28°C in Silverson Blender for 15s and then in Potter Elvehjem for 15s (3 strokes). 37.5 ml of the homogenate was applied to a linear sucrose gradient of 1000 ml from 0.5M to 2.26M and 200 ml 2.62 M-sucrose as a cushion. The gradient contained 10^{-4} M-chloroquine + 1mM- NaHCO_3 . The Sorvall SZ-14 zonal rotor and the solutions were maintained at 28°C. The gradient and the homogenate were applied to rotor spinning at 2,500 rev/min ($\cong 343g$). The rotor speed was increased to 12,000 rev/min ($\cong 8,000g$) after the homogenate had been applied and was spun for 30 min. The contents of the rotor were emptied by peristaltic pump. Fractions of about 12 ml were collected. The contents of tubes were mixed before visual observation by phase contrast microscopy, $^{45}\text{Ca}^{2+}$ and DNA estimation, enzyme assays and sucrose concentration were determined (1.8 and 1.9).

+ ————— +	alkaline phosphatase (Al.P.) (right hand scale)
Δ ————— Δ	deoxyribose nucleic acid (D.N.A.) (left hand scale)
∇ - - - - - ∇	5' Nucleotidase (N) (left hand scale)

FIGURE 4B

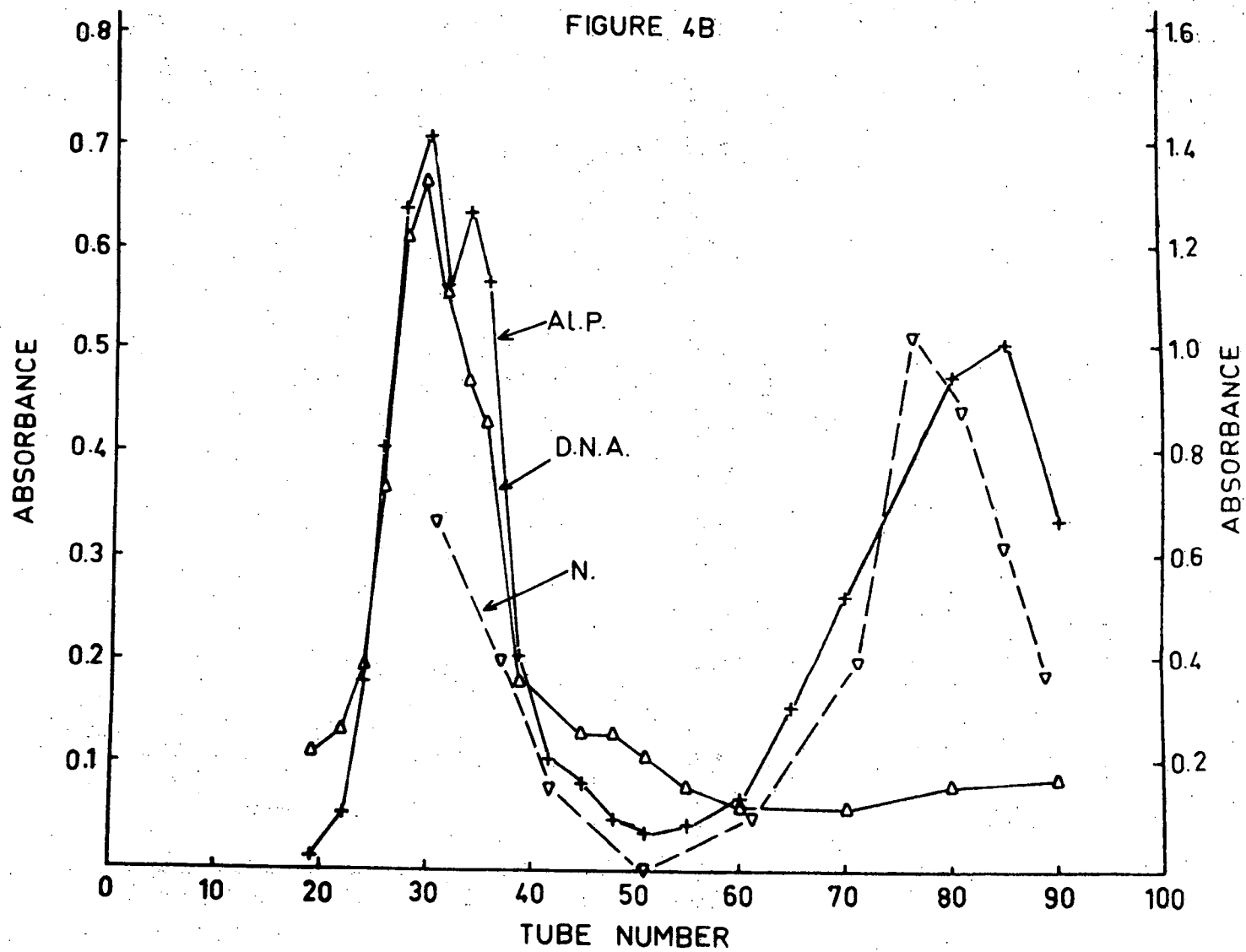


FIGURE 5A

Zonal rotor fractionation at 0°C

4 rachitic chick duodena were loaded with 500 μ l $^{45}\text{Ca}^{2+}/^{40}\text{Ca}^{2+}$ solution (2 mg, 7.5 μ Ci) for 20 min (1.4.1 and 1.4.2). The mucosal cells were isolated (1.5.1) and homogenized in 0.25M-sucrose + 1mM- NaHCO_3 + 2mM- Mg^{2+} + 10^{-4} M-chloroquine at 0°C in Silverson Blender for 15s and then in Potter Elvehjem for 15s (3 strokes). 35 ml of the homogenate was applied to a linear sucrose gradient of 1000 ml from 0.5M to 2.26M and 200 ml 2.62M-sucrose as a cushion. The gradient contained 10^{-4} M-chloroquine + 1mM- NaCl . The Sorvall SZ-14 zonal rotor and the solutions were maintained at 0°C. The gradient and the homogenate were applied to the rotor spinning at 2,500 rev/min (\cong 343g). The rotor speed was increased to 12,000 rev/min (\cong 8,000g) after the homogenate had been applied and spun for 30 min. The contents of the rotor were emptied by peristaltic pump. Fractions of about 12 ml were collected. The contents of the tubes were mixed before visual observation by phase contrast microscopy, $^{45}\text{Ca}^{2+}$ and DNA estimation, enzyme assays and sucrose concentration were determined (1.8 and 1.9).

- — — — ● sucrose gradient (S.G.) (left hand scale)
- 0 ————— 0 acid phosphatase (Ac.P.) (left hand scale)
- X - - - - X succinic dehydrogenase (S.D.) (left hand scale)
- ————— □ $^{45}\text{Ca}^{2+}$ (right hand scale)

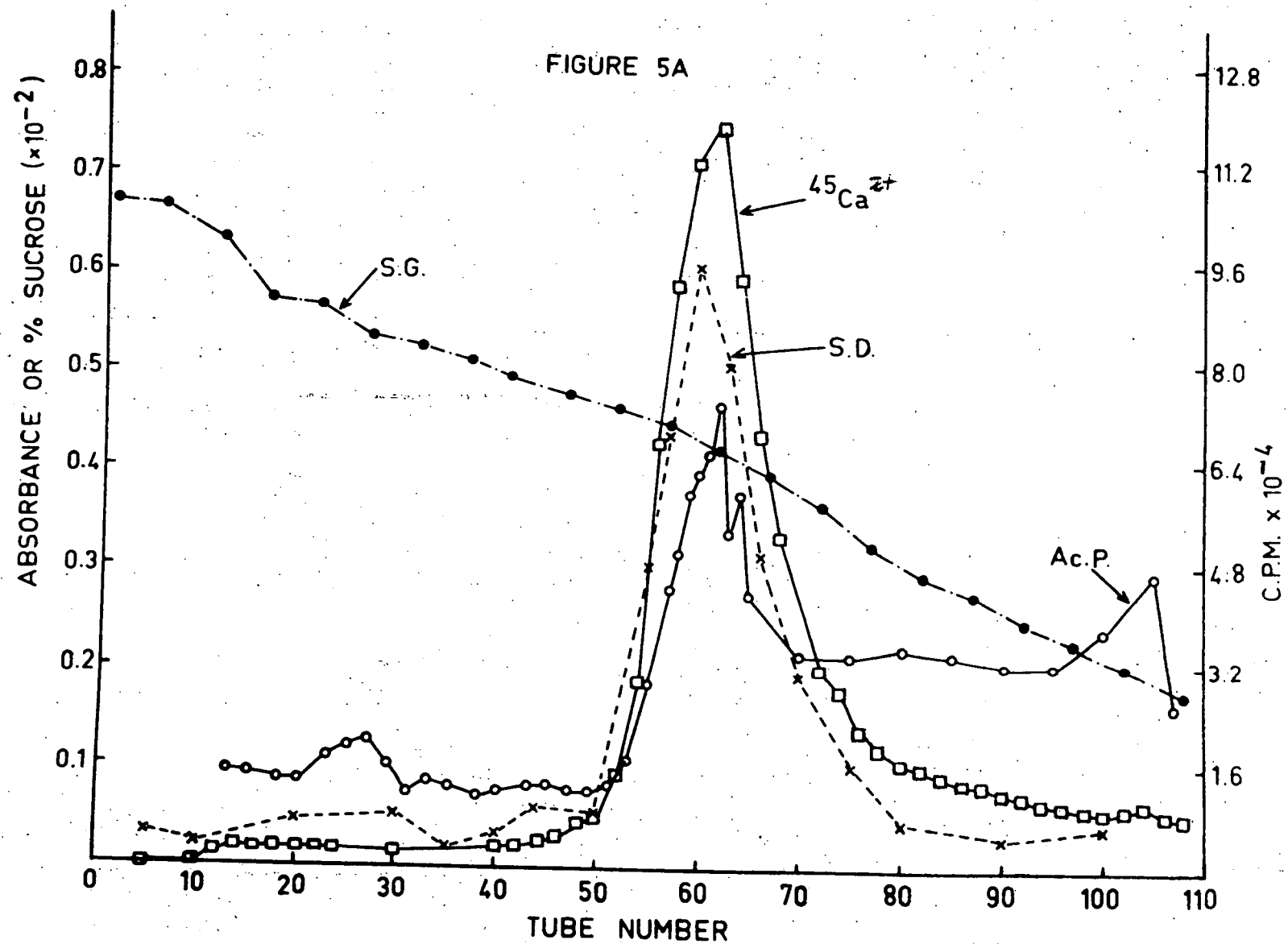
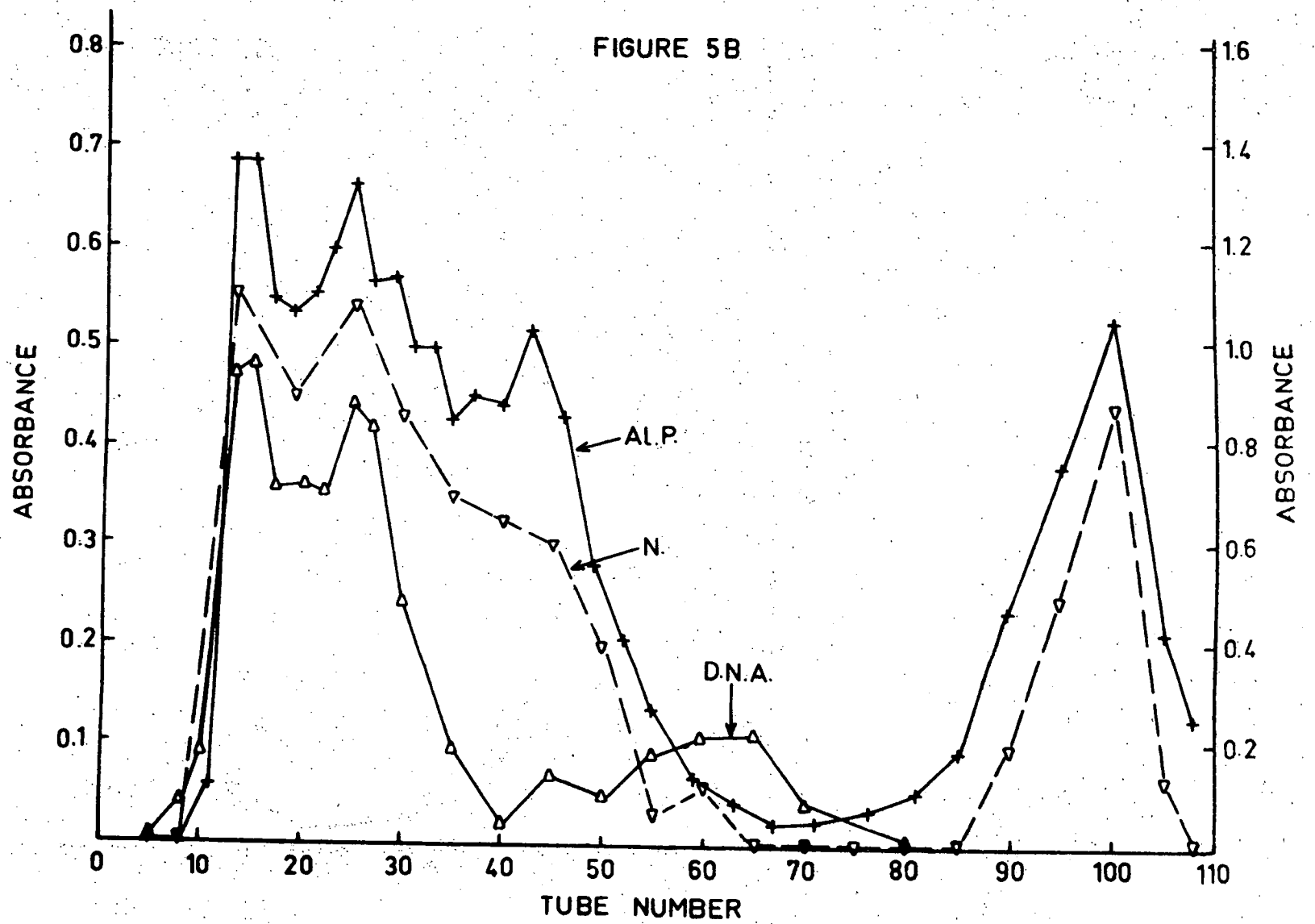


FIGURE 5B

Zonal rotor fractionation at 0°C

4 rachitic chick duodena were loaded with 500 μ l $^{45}\text{Ca}^{2+}/^{40}\text{Ca}^{2+}$ solution (2 mg, 7.5 μ Ci) for 20 min (1.4.1 and 1.4.2). The mucosal cells were isolated (1.5.1) and homogenized in 0.25M-sucrose + 1mM- NaHCO_3 + 2mM- Mg^{2+} + 10^{-4} M-chloroquine at 0°C in Silverson Blender for 15s and then in Potter Elvehjem for 15s (3 strokes). 35 ml of the homogenate was applied to a linear sucrose gradient of 1000 ml from 0.5M to 2.26M and 200 ml 2.62M-sucrose as a cushion. The gradient contained 10^{-4} M-chloroquine + 1mM- NaCl . The Sorvall SZ-14 zonal rotor and the solutions were maintained at 0°C. The gradient and the homogenate were applied to the rotor spinning at 2,500 rev/min (\cong 343g). The rotor speed was increased to 12,000 rev/min (\cong 8,000g) after the homogenate had been applied and spun for 30 min. The contents of the rotor were emptied by peristaltic pump. Fractions of about 12 ml were collected. The contents of the tubes were mixed before visual observation by phase contrast microscopy, $^{45}\text{Ca}^{2+}$ and DNA estimation, enzyme assays and sucrose concentration were determined (1.8 and 1.9).

+ ————— + alkaline phosphatase (Al.P.) (right hand scale)
 Δ ————— Δ deoxyribose nucleic acid (D.N.A.) (left hand scale)
 ∇ - - - - - ∇ 5' Nucleotidase (N.) (left hand scale)



Discussion

In chapter 2 it was shown that mucosal cells loaded with $^{45}\text{Ca}^{2+}$ in vivo and homogenized in 0.25M-sucrose at 0°C, had most of the $^{45}\text{Ca}^{2+}$ in the mitochondrial fraction. It may be that the conditions used lead to the uptake of $^{45}\text{Ca}^{2+}$ by the mitochondria. When a cell homogenate was prepared, then $^{45}\text{Ca}^{2+}$ added at 0°C, all the added $^{45}\text{Ca}^{2+}$, 400 µg/diudenum, was taken up—largely by the mitochondrial (Table 13). Thus the organelles of diudenum mucosal cells have the capacity to take up completely amounts of calcium normally present in mucosal cells during calcium absorption. Isolated mitochondria suspended in 0.25M-sucrose (not vigorously washed since it was intended to stimulate conditions in the homogenate) would accumulate approximately 2 µg Ca^{2+} /mg protein and no significant difference was observed between mitochondria from rachitic or vitamin D₃-replete chicks. These amounts of Ca^{2+} compare with amounts of Ca^{2+} found in mitochondria isolated from mucosal cells during Ca^{2+} absorption (cf. chapter 2).

Attempts were made to find inhibitors which would prevent the uptake of Ca^{2+} by mitochondria. The ideal inhibitor would completely inhibit uptake of Ca^{2+} without causing release of endogenous Ca^{2+} of the organelles. No such inhibitor was found. Inhibitors of oxidative phosphorylation partially inhibited uptake but caused a large efflux of Ca^{2+} from mitochondria eg DNP and dicoumarol and therefore could not be used to find the true distribution of Ca^{2+} in the cell. The respiratory inhibitors antimycin, rotenone and warfarin were less effective inhibitors of Ca^{2+} uptake at 0°C but did not cause release of Ca^{2+} from mitochondria. These inhibitors are known to inhibit Ca^{2+}

uptake during accumulation at 30°C in the presence of substrates (DeLuca and Engstrom, 1961; Engstrom and DeLuca, 1964b; Carafoli et al, 1966) and were used in some experiments to inhibit mitochondrial uptake in the live chick and prevent redistribution during fractionation. However these inhibitors did not alter the pattern of $^{45}\text{Ca}^{2+}$ distribution (Tables 27 and 28) where mitochondria contain most of the Ca^{2+} and the supernatant contained very little soluble Ca^{2+} . It became obvious that one of the most important parameters affecting Ca^{2+} redistribution is the cooling of mucosal cells and homogenation at 0°C when the mitochondria can avidly take up any soluble Ca^{2+} . The inhibitors antimycin, rotenone and warfarin are not very effective inhibitors of this process and therefore the results of Tables 27 and 28 were to be expected.

Sodium fluoride that inhibited Ca^{2+} uptake by isolated mitochondria had little effect on the pattern of calcium distribution in mucosal cells (Table 25). However it was not examined further, because it was suspected that if a substantial amount of Ca^{2+} did occur in the supernatant, then this Ca^{2+} would precipitate as CaF_2 since the solubility product of CaF_2 is 3×10^{-11} (Vogel, 1948).

Slater and Cleland (1952) introduced EDTA into media for the preparation of mitochondria and observed that EDTA reduced the amount of Ca^{2+} taken up by the mitochondria. The more specific chelating agent EGTA inhibited Ca^{2+} uptake by isolated mitochondria and caused only slight loss of (endogenous) Ca^{2+} from previously loaded mitochondria (Tables 16 and 18). This would suggest that EGTA would be an excellent agent for preventing the redistribution of Ca^{2+} during

homogenation. However when EGTA was added to a previously $^{45}\text{Ca}^{2+}$ loaded cell homogenate which contained only 2% $^{45}\text{Ca}^{2+}$ in the soluble phase, 14% of the total $^{45}\text{Ca}^{2+}$ was found in the final supernatant on fractionation (Table 29). This result suggests that under the conditions present in the homogenate some $^{45}\text{Ca}^{2+}$ is released from the organelles by EGTA and is found in the soluble fraction. Table 34 also shows an amount of approximately 15% of the total $^{45}\text{Ca}^{2+}$ was released by EGTA at 0°C into the soluble phase. When EGTA was used during the isolation and homogenation of mucosal cells that had been loaded with $^{45}\text{Ca}^{2+}$ in vivo, up to one third of the cell Ca^{2+} was found in the soluble fraction (Table 30). These experiments suggest that the cytoplasmic Ca^{2+} is a greater proportion of the total Ca^{2+} than previously accepted (Thiers and Vallee, 1957; Vasington and Murphy, 1962; Drahota et al, 1965; Wester, 1965), but due to the uncertainties of the effect of EGTA on releasing some Ca^{2+} into the soluble phase, the precise figure cannot be obtained by this method.

One substance that has a great use for inhibiting Ca^{2+} uptake by mitochondria at 0°C and 30°C is ruthenium red. This histological stain for mucopolysaccharides was shown by Moore (1971) to inhibit energy dependent Ca^{2+} uptake by rat liver mitochondria and more recently Vasington et al (1972), who showed that ruthenium red combines with the high and low affinity binding sites for Ca^{2+} of rat liver mitochondria. Moore (1971) and Vasington et al (1972) have quoted values of 2-4 nmoles of ruthenium red/mg mitochondrial protein would inhibit uptake of Ca^{2+} by mitochondrial. In experiments done in this chapter an excess of ruthenium red was used in prevention of uptake of Ca^{2+}

by mitochondria (1000 nmoles/mg protein) and by the homogenate (100 nmoles/mg protein). Ruthenium red prevented Ca^{2+} uptake by chick intestinal mitochondria at 0°C and 30°C (Tables 16 and 17) and did not cause Ca^{2+} release from previously loaded mitochondria (Tables 18 and 19A). When ruthenium red was added to an homogenate of mucosal cells at 0°C it prevented uptake of added $^{45}\text{Ca}^{2+}$ by the organelles and left most of the Ca^{2+} in the supernatant (Table 24). Thus this agent seemed to be the inhibitor sought, but it had a defect in that it combined with and caused many organelles and proteins to centrifuge down at low speeds, making it impossible to study the distribution of Ca^{2+} in these organelles. When mucosal cells, loaded with $^{45}\text{Ca}^{2+}$ in vivo, were homogenized at 0°C in sucrose containing ruthenium red, the soluble phase contained 15% of the total $^{45}\text{Ca}^{2+}$ (Table 31A), and similar amounts of soluble Ca^{2+} were found in fed and starved chick duodenum mucosal cells (Table 31B).

When mucosal cells loaded with $^{45}\text{Ca}^{2+}$ were isolated and homogenized in 0.25M-sucrose at 0°C , then allowed to come to 22°C , there was an increase in the soluble $^{45}\text{Ca}^{2+}$ from 10% at 0°C to 31% at 22°C (Table 33). These observations are reminiscent of an experiment done by Rasmussen et al (1963) with rat intestinal villi that had taken up $^{45}\text{Ca}^{2+}$ at 0°C and released most of this $^{45}\text{Ca}^{2+}$ on warming to 33°C . Thus cooling a cell to 0°C may completely distort the pattern of Ca^{2+} distribution within the cell. The conventional estimates of the free soluble Ca^{2+} in cells were obtained in this way (Thiers and Vallee, 1957; Vasington and Murphy, 1962; Drahota et al, 1965; Wester, 1965). It seems possible that these estimates may be in error because of the

avid uptake of Ca^{2+} by mitochondria at 0°C . For this reason attempts were made to isolate and homogenize mucosal cells at 22°C or above both in the presence and absence of inhibitors. When cells were scraped and homogenized at 22°C (Table 33), then 13% of the total Ca^{2+} was found in the supernatant. This experiment examined the soluble phase for enzymes of mitochondria and lysosomes to show that homogenation at 22°C did not cause any great damage to these organelles. The estimate of 13% for the soluble Ca^{2+} may be too low since no inhibitors were present to prevent Ca^{2+} uptake by mitochondria. When ruthenium red was present in the homogenation media at 22°C , then 32% of the total Ca^{2+} was found in the final supernatant of both rachitic and vitamin D_3 -replete chick duodena (Table 38). It has already been shown that ruthenium red prevents Ca^{2+} uptake but does not cause release from organelles at 30°C (Tables 17 and 19A). When, in addition to homogenizing the cells at 22°C in the presence of ruthenium red, the chicks were given rotenone plus warfarin in vivo, the amount of soluble Ca^{2+} found rose to 44% (Table 38). However these inhibitors may have inhibited the mechanism that pumps Ca^{2+} out of the cell, eg. in Table 28 as antimycin plus warfarin given in vivo to chicks led to a greater accumulation of total Ca^{2+} within the cells of vitamin D-replete chicks than the rachitic chick cells, which is contrary to the usual situation. Thus the estimate of 32% of Ca^{2+} in the supernatant seems more correct.

A further attempt to find the concentration of Ca^{2+} in the cytoplasm was made during the zonal centrifuge. In this centrifuge the homogenate was applied on to a sucrose density gradient spinning at 350g. The centrifuge was then immediately accelerated to 8,000g

and under these conditions the organelles were rapidly removed from the soluble phase. When previously loaded cells were homogenized in 0.25M-sucrose at 0°C and 28°C, then immediately placed on the zonal centrifuge at the appropriate temperature, it was found that for cells homogenized at 0°C the mitochondria had accumulated 72% of the total $^{45}\text{Ca}^{2+}$, whereas for cells treated at 28°C the amount was 44%. Since only small amounts of $^{45}\text{Ca}^{2+}$ were associated with nuclei, or lysosomes, or endoplasmic reticulum then the amount of soluble Ca^{2+} of cells homogenized at 28°C could have been one third of the total Ca^{2+} as found above (Table 38).

The conclusion drawn from the work reported in this chapter is that the amount of Ca^{2+} in the cytoplasm may be greater than previously thought. During absorption of calcium, and in normally fed or starved chicks, the cytoplasmic Ca^{2+} was found to be approximately one third of the total Ca^{2+} content of the mucosal cell. However mitochondria still contained a large proportion of the cellular Ca^{2+} . Thus this indicates that mitochondrial Ca^{2+} is in equilibrium with the cytoplasmic Ca^{2+} in the live chick intestinal cell. This equilibrium may be upset by the removal of the duodenum from the chick, and by cooling the cell prior to homogenation at 0°C and even at 28°C.

In support of this idea of a concentration of 1-2mM- Ca^{2+} within mucosal cells, are the observations made during several experiments with non-everted sacs of duodena. The Ca^{2+} content of the non-everted sacs from starved chicks actually decreased when the sacs were incubated under 95% O_2 / 5% CO_2 for 30 min at 30°C in

Krebs-Henseleit bicarbonate + 20mM-glucose + 1mM- Ca^{2+} . The results of such an experiment are shown below and show that the tissue lost Ca^{2+} to the medium. The results are the average for 24 determinations.

Type of chick	Ca^{2+} present in the mucosal cell of duodenum	
	Before incubation Ca^{2+} $\mu\text{g/g}$ wet weight tissue	After incubation Ca^{2+} $\mu\text{g/g}$ wet weight tissue
Vitamin D_3 -treated	102	73
Rachitic	96	44

CHAPTER 4

THE SUBCELLULAR DISTRIBUTION AND LOCATION OF A CALCIUMBINDING PROTEINIntroduction

In 1966, Wasserman and Taylor reported the existence of a protein in the intestinal mucosa of vitamin D-replete chicks that was present in much smaller amounts in rachitic chicks, and this protein had the property of combining with Ca^{2+} in vitro. This calcium binding protein (CaBP) appeared in the mucosal cells within 5 h of giving 5,000 I.U. of vitamin D_3 to rachitic chicks (Ebel, Taylor and Wasserman, 1969), and the time course of appearance of CaBP was correlated with the increased rate of translocation of Ca^{2+} which followed treatment with vitamin D. No explanation of the role of CaBP had been put forward. A large proportion of the Ca^{2+} of the intestinal mucosa is present in the mitochondria as shown in chapters 2 and 3. Furthermore it has been shown that in vivo the turnover by the vitamin D_3 treated mucosal cells is greater than the rachitic cells (chapter 2). This turnover of $^{45}\text{Ca}^{2+}$ was found to occur more rapidly in mitochondria from vitamin D_3 -replete tissue.

Before a role for CaBP could be postulated it was necessary to know its whereabouts in the cell, i.e., externally or in the organelles or in the cytoplasm. In this chapter an attempt was made to locate the CaBP in the subcellular fractions and to see whether CaBP could be found in fluid bathing the microvilli.

PART 14.1 The location of the calcium binding proteinPart 1.14.1.1 An attempt to locate the calcium binding protein in the lumenMETHOD

Rachitic and vitamin D-replete (800 I.U. vitamin D₃ administered 65 h before the experiment) chicks were used to prepare a calcium binding protein extract. Firstly after washing out the duodenum, 500 µl 0.154M-NaCl was placed in the duodenum for 20 min as described for ⁴⁵Ca²⁺ loading in 1.4.1 and 1.4.2. Then the loop was washed out with 5 ml 0.154M-NaCl. The washings were collected. The cells were isolated, homogenized in Tris buffer of Wasserman and Taylor (1966) and centrifuged at 14,000g for 10 min. The supernatant was centrifuged at 78,000g for 30 min. The washings and the cell supernatant were then tested for Ca²⁺ binding by the method described in 1.8.5. The washings and the cell supernatant were then concentrated at least 8 fold by use of ultrafiltration apparatus (Diaflo apparatus, UM 10 membrane) and again a Ca²⁺ binding estimation was performed.

RESULTS

In Table 39 one can observe that no Ca²⁺ binding activity is present in the luminal contents plus washings after 20 min contact within the lumen. The homogenate of the mucosal cells of both rachitic and vitamin D-replete chicks contained binding activity with a greater amount for vitamin D tissue.

TABLE 39

An attempt to locate the calcium binding protein in the lumen

The duodenal loops of vitamin D₃-replete (800 I.U. vitamin D₃ given 65 h before the experiment) and rachitic chicks were loaded with 500 µl 0.154M-NaCl for 20 min. The contents of the loops and the 5 ml of 0.154M-NaCl of washings were collected. The mucosal cells were isolated, homogenized in Tris buffer (Wasserman and Taylor, 1966) and centrifuged at 14,000g for 10 min and the supernatant was centrifuged at 78,000g for 30 min. The washings and the cell supernatant were tested for Ca²⁺ binding as described in 1.8.5. The washings and the cell supernatant were then concentrated at least 8 fold by ultrafiltration through Diaflo UM 10 membrane and again a Ca²⁺ binding activity was tested. The results are expressed as nmoles ⁴⁰Ca²⁺ bound per 100 mg protein.

Type	Level of Ca ²⁺ used in test M	<u>⁴⁰Ca²⁺ bound nmoles/100 mg protein</u>			
		Lumen contents and washings		Supernatant of cell homogenate	
		Rachitic	Vitamin D ₃ - treated	Rachitic	Vitamin D ₃ - treated
not conc.	10 ⁻⁴	none detected	none detected	66.1	100.9
conc.	10 ⁻⁵	none detected	none detected	20.4	54.4

Part 1.24.1.2. Intracellular location of calcium binding protein

In part 1.1 the CaBP was shown to be present in the cellular extract of the intestine and not in the luminal side of microvilli or is not loosely bound on the outer surface of the mucosal cell. In this part the intracellular location of this CaBP was determined.

METHOD

For the intracellular location of CaBP, cell homogenates of rachitic and vitamin D-replete chick mucosal cells were prepared in Tris buffer (Wasserman and Taylor, 1966) and in 0.25M-sucrose. The Tris buffer homogenate was centrifuged at 78,000g for 10 min, the homogenate and the supernatant were tested for Ca^{2+} binding as described in 1.8.5. The sucrose homogenate was fractionated and treated as described in 1.5.3.1 and 1.5.4.2. The Ca^{2+} binding activity of these fractions was estimated by the chelex test as described in 1.8.5.

RESULTS

The results shown in Table 40 confirm that vitamin D_3 -treated chicks possess more CaBP in the homogenate of mucosal cells than rachitic chicks. The homogenate prepared with Tris buffer increased in specific activity when the particulate matter was removed by centrifugation at 78,000g. When the homogenate was prepared with 0.25M-sucrose and fractionated, the final supernatant contained most of the CaBP as shown in Table 41. The greatest difference in binding activity between the 2 groups resides in the final supernatant. The isolated organelles were homogenized with

the Tris buffer and centrifuged. The supernatant from the organelles contained the same binding activity in both rachitic and vitamin D-treated groups.

TABLE 40

Calcium binding protein in mucosal cells

Mucosal cells from the duodena of rachitic and vitamin D₃-replete chicks (800 I.U., 48 h previously) were homogenized in Tris buffer (Wasserman and Taylor, 1966) and tested for binding activity as described in 1.8.5. The homogenate was centrifuged at 78,000g for 10 min and the supernatant tested in the same way. The results are expressed as nmoles ⁴⁰Ca²⁺ bound per 100 mg protein (mean of three experiments).

Final Conc. of ⁴⁵ Ca ²⁺	Type of Chick	⁴⁰ Ca ²⁺ bound nmoles/100 mg Protein	
		homogenate	supernatant
10 ⁻⁵ M	Vitamin D ₃ - treated	7.0	13.8
	Rachitic	3.6	6.3
10 ⁻⁴ M	Vitamin D ₃ - treated	54.0	102.0
	Rachitic	28.0	38.0
10 ⁻³ M	Vitamin D ₃ - treated	270.0	-
	Rachitic	16.0	-

TABLE 41

Location of calcium binding protein in mucosal cells

These fractions were prepared in 0.25M-sucrose as described in 1.5.3.1 and 1.5.4.2 and then extracted for calcium binding protein with Tris buffer (Wasserman and Taylor, 1966).

The extracts were tested for binding protein at a final concentration of 10^{-4} M $^{45}\text{Ca}^{2+}/^{40}\text{Ca}^{2+}$ solution. The results were calculated to show the binding of the fractions obtained from one duodenum as $^{40}\text{Ca}^{2+}$ bound nmoles/duodenum.

Subcellular fractions	$^{40}\text{Ca}^{2+}$ bound nmoles/duodenum	
	Rachitic	Vitamin D ₃ -treated
Nuclei	13.6	12.2
Mitochondria		
Top	none	none
Middle	none	none
Heavy	14.0	23.2
Microsomes		
Light & Heavy	32.0	30.0
Final Supernatant	52.8	94.0

Part 1.34.1.3 To determine whether calcium binding protein is present in mitochondria

In part 1.2 some Ca^{2+} binding activity was shown in the extracts of mitochondria. These mitochondria may have been contaminated with cytoplasmic components. Therefore an experiment was done in which the mitochondria were prepared from rachitic and vitamin D-replete chicks.

METHOD

Mitochondria were prepared from duodenal mucosal cells of rachitic and vitamin D_3 -replete chicks (treated 68 h previously) and treated as described in 1.5.3.1.2 and 1.5.4.3.

RESULTS

Even after washing with sucrose, the mitochondria contained some Ca^{2+} binding activity that could be solubilised by treating with low ionic strength Tris buffer. No difference in amount of binding was associated with vitamin D status (Table 42). The cytoplasmic proteins of the cell again contained most of the Ca^{2+} binding activity, and there was a marked difference after treatment with vitamin D (Table 42).

Since these experiments were done Lehninger has shown that mitochondria contain a calcium binding protein that possesses a higher molecular weight than CaBP isolated by Wasserman and Taylor (1966) (Lehninger and Carafoli, 1969; Carafoli, Hansford, Sacktor and Lehninger, 1971).

TABLE 42

To verify whether calcium binding protein is associated with the mitochondria.

800 I.U. vitamin D₃ were given to rachitic chicks 68 h before the experiment. Mitochondria from rachitic and vitamin D₃-replete chicks were prepared in 0.25M-sucrose and then extracted with Tris buffer (Wasserman and Taylor, 1966) as described in 1.5.4.3. The mitochondrial extract from 50 vitamin D₃-replete and 52 rachitic chicks, and the cell homogenate supernatant (after spinning at 78,000g for 30 min), were tested for Ca²⁺ binding activity by chelex 100 test as described in 1.8.5. The results are the average of duplicate determination and expressed as nmoles per 100 mg protein.

Fraction	Type of Chick	Conc. of $\frac{^{45}\text{Ca}^{2+}}{^{40}\text{Ca}^{2+}}$ solution M	$^{40}\text{Ca}^{2+}$ bound nmoles/100 mg protein
Mitochondrial extract	Vitamin D ₃ -treated	10 ⁻⁴	14.8
	Rachitic	10 ⁻⁴	12.2
Cell supernatant	Vitamin D ₃ -treated	10 ⁻⁴	94.9
	Rachitic	10 ⁻⁴	15.2

PART 24.2 The effect of ruthenium red of calcium binding to calcium binding protein

In part 1 the subcellular location was ascertained by fractionation to be cytoplasmic. However during homogenation the protein may be dislodged from organelles, possibly the mitochondria or brush borders.

Thus in part 2 the effect of ruthenium red on the Ca^{2+} binding power of this protein was examined, first of all by loading the gut with ruthenium red and homogenizing these isolated cells in Tris buffer; secondly by isolating these cells and homogenizing and then treating the homogenate with ruthenium red. Thirdly the isolated crude CaBP was treated with this substance to determine its effect on the Ca^{2+} binding to this protein.

Part 2.14.2.1 The effect of ruthenium red on calcium binding by calcium binding proteinMETHOD

800 I.U. of vitamin D₃ in propylene glycol were given to rachitic chicks intramuscularly 72 h before the experiment.

The duodenal loops of vitamin D₃-replete chicks were loaded with 500 μ l 0.154M-NaCl, with or without 0.7 mg ruthenium red, for 20 min as described in 1.4.1 and 1.4.2. The mucosal cells were isolated as described in 1.5.1. A homogenate was prepared in Tris buffer pH 7.1 (Wasserman and Taylor, 1966) as described in 1.5.2. The homogenate of mucosal cells loaded with saline was divided in half (total volume 20 ml), to one half was added 1 ml 0.25M-sucrose + 50mM-Tris Tricine pH 7.2 + 14 mg ruthenium red and to the other half was added 1 ml 0.25M-sucrose + 50mM-Tris Tricine pH 7.2.

The 3 homogenates were centrifuged at 78,000g for 30 min. The supernatant of the homogenate were treated for Ca²⁺ binding as described in 1.8.5 using 1 ml sample.

RESULTS

Ruthenium red does not appear to effect the Ca²⁺ binding by CaBP by either treatment (Table 43).

TABLE 43

The effect of ruthenium red on calcium binding by calcium binding protein

800 I.U. vitamin D₃ were given to rachitic chicks intramuscularly 72 h before the experiment. Duodenal loops were loaded with 500 μ l 0.154M-NaCl with or without 0.7 mg ruthenium red for 20 min as described in 1.4.1 and 1.4.2. The mucosal cells were isolated as described in 1.5.1. Homogenates were prepared as described in 1.5.2. The homogenate prepared from duodenal loops loaded with 0.154M-NaCl was divided in half (total volume 20 ml). To one half was added 1 ml 0.25M-sucrose + 50mM-Tris Tricine + 14 mg ruthenium red. These homogenates were centrifuged at 78,000g for 30 min. The supernatants were chelex tested for Ca²⁺ binding (1.8.5) and estimated for protein (1.8.1). The results are expressed as ⁴⁰Ca²⁺ bound nmoles per 100 mg protein.

Treatment	Conc. of test ⁴⁵ Ca ²⁺ / ⁴⁰ Ca ²⁺ solution M	⁴⁰ Ca ²⁺ bound nmoles/100 mg protein
0.154M-NaCl only	10 ⁻⁴	161.0
0.154M-NaCl loaded and 14 mg ruthenium red added to homogenate	10 ⁻⁴	126.0
0.154M-NaCl + 0.7 mg ruthenium red loaded	10 ⁻⁴	154.8

Part 2.24.2.2. The effect of ruthenium red on the isolated calcium binding proteinMETHODS

To 1 ml of crude CaBP (prepared as described in 1.5.4.3) was added 100 μ l $^{45}\text{Ca}^{2+}/^{40}\text{Ca}^{2+}$ solution (100 nmoles, 0.25 μ Ci) + 100 μ l 0.25M-sucrose or 0.25M-sucrose + 1.4 mg ruthenium red. The mixtures were sampled before and after ultrafiltration through UM 2 Diaflo membrane (by syringe). The mixtures and filtrate were counted.

RESULTS

The amount of calcium bound by the CaBP is the same in the presence and absence of ruthenium red (Table 44). Thus ruthenium red has had no effect on the amount of Ca^{2+} bound/100 mg protein.

TABLE 44

The effect of ruthenium red on calcium binding by calcium binding protein

To 1 ml crude CaBP (prepared as described in 1.5.4.1) was added 100 μ l $^{45}\text{Ca}^{2+}/^{40}\text{Ca}^{2+}$ solution (100 nmoles, 0.25 μCi) + 100 μ l 0.25M-sucrose + 1.4 mg ruthenium red or 0.25M-sucrose. The mixtures were filtered through UM 2 Diaflo membranes (by syringe). The results are the average of duplicate determinations and are expressed as $^{40}\text{Ca}^{2+}$ bound nmoles per 100 mg protein.

Inhibitor	$^{40}\text{Ca}^{2+}$ bound nmoles/100 mg protein
none	109.5
ruthenium red	111.3

Discussion

Evidence for the induction of CaBP synthesis by vitamin D₃ is impressively reviewed by Wasserman and Corradino (1971). The method used by Wasserman and Taylor (1966) to extract CaBP was homogenation in low ionic strength Tris buffer which would disrupt many of the cell structures. Thus this method of extraction does not lead to information about the location of CaBP in the cell. Taylor and Wasserman, (1970a) by the use of fluorescent antibody to CaBP reported that CaBP was located at the brush borders and the goblet cells. If this were so then it would be expected that the fluid bathing the luminal surface would contain some of this CaBP, but none could be detected. In the work reported in this chapter mucosal cells were homogenized in 0.25M-sucrose and the homogenate fractionated in this medium. Most of the CaBP was found in the 78,000g supernatant which suggests its location is in the cytoplasm. However it is possible that even this treatment in isotonic sucrose has released CaBP from some membrane structure. Certainly the brush borders are largely destroyed by homogenation in 0.25M-sucrose. The only method of preparing brush borders uses EDTA thus making it impracticable to test for CaBP (Forstner, Sabesin and Isselbacher, 1968).

Ca²⁺ binding activity was found in small amount in nuclei, mitochondria and microsomes (but there was no difference due to vitamin D status) and may reflect binding of Ca²⁺ by other protein than the CaBP. The uptake of Ca²⁺ by mitochondria is inhibited by ruthenium red (Moore, 1971; Vasington, Gazzotti, Tiozzi and Carafoli, 1972). Carafoli, Gazzotti, Vasington, Sottocasa, Sandri, Panfili and

de Bernard (1972) found that the protein responsible for high affinity Ca^{2+} binding of mitochondria combines with ruthenium red. The CaBP prepared by Wasserman and Taylor (1966) method would still combine with Ca^{2+} even in the presence of a large excess of ruthenium red. This is in accord with the observation reported later in 6.4 that ruthenium red does not interfere with the in vivo absorption of Ca^{2+} . The uptake of Ca^{2+} by mitochondria and the passage of Ca^{2+} across the brush border appear to be a different process by these observations.

Thus the CaBP appears to be located in the cytoplasm of the mucosal cell, or if it is located in the microvillous region, it is easily released by homogenation, although little of the CaBP finds its way to the lumen.

CHAPTER 5THE EFFECT OF VARIOUS FACTORS ON CALCIUM UPTAKE AND RELEASEBY MITOCHONDRIAIntroduction

In Chapter 2 evidence has been produced that mitochondria may play a role in the translocation of Ca^{2+} across the mucosal cell. If mitochondria accumulate Ca^{2+} then some process of release from these organelles must be present and may be influenced by the presence of vitamin D. This chapter examines some of the substances likely to be involved in such a process.

DeLuca and co-workers (DeLuca and Engstrom, 1961; DeLuca, Engstrom and Rasmussen, 1962; Engstrom and DeLuca, 1962, 1964a) reported on the characteristic of Ca^{2+} release from mitochondria from liver, kidney and intestine of rats that had previously been treated with vitamin D. These mitochondria released more $^{45}\text{Ca}^{2+}$ than mitochondria from vitamin D-deficient. PTH accelerated this release but only from vitamin D-replete rat kidney mitochondria. An investigation was done to determine the effect of PTH on chick intestine mitochondria.

Neville and Holdsworth (1969) using chick intestine implicated adenosine 3', 5' cyclic monophosphate (cyclic AMP) as one of the messengers in the Ca^{2+} translocation chain. Thus the effect of cyclic AMP and related adenine nucleotides were tested for their effects on the release of $^{45}\text{Ca}^{2+}$ from mitochondria.

Prostaglandins have become a topic of increasing interest, so it was decided that one should observe the effect of these compounds

on Ca^{2+} release from mitochondria.

The CaBP was shown in the previous chapter to be loosely bound or was actually present in the cytoplasm. Therefore it could be considered as a method of release of Ca^{2+} from the mitochondria. This proposal was studied along with the effect of CaBP on uptake and release of Ca^{2+} from mitochondria.

PART 15.1 The effect of parathyroid hormone, adenine nucleotides, EDTA, prostoglandin and calcium binding protein on calcium release from mitochondriaGENERAL METHOD

The procedure followed in this part is set out in 1.6.3. The mitochondria loaded with $^{45}\text{Ca}^{2+}$ were prepared in the manner described in 1.5.3.3. The mitochondria were resuspended in 0.25M-sucrose + 50mM-Tris Tricine pH 7.1 with or without 10 mg/ml BSA and adjusted to a mitochondrial protein content between 3 to 6 mg/ml. The mitochondrial suspension was added to a medium containing 0.164M-sucrose, + 62mM-imidazole + 56mM-succinate at pH 7.1 with or without 10 mg/ml BSA plus substance to be tested or 0.25M-sucrose, with or without 50mM-Tris Tricine, with or without 10 mg/ml BSA, plus substance to be tested. The release of $^{45}\text{Ca}^{2+}$ was measured by the centrifugation (1.6.2.1) or by filtration (1.6.2.2) methods. The results are expressed as % of total $^{45}\text{Ca}^{2+}$ released.

Part 1.15.1.1 The effect of adenine nucleotides on release of calcium from mitochondriaMETHOD

The mitochondria were resuspended in 0.25M-sucrose and adjusted to mitochondrial protein content 3.5 mg/ml. 2 ml of the mitochondrial suspension were added to 2 ml of medium containing 0.25M-sucrose + 32mM-imidazole + 28mM-succinate at pH 7.1 + 5 mg BSA + 2×10^{-4} M adenine nucleotide and incubated at 0°C and 30°C for 40 min. 1 ml samples were taken from the mixtures at 10 min intervals up to 40 min and centrifuged at 20,000g for 10 min at the appropriate temperature. The supernatant and the residue were counted. The results are expressed as percent release of $^{45}\text{Ca}^{2+}$ from mitochondria.

RESULTS

The adenine nucleotides that were used were adenosine triphosphate, adenosine monophosphate, adenosine 3', 5' cyclic monophosphate and N-6-2-O-dibutyryl adenosine 3', 5' cyclic monophosphate. Samples were taken from the incubation mixture for 40 min but no effect on release was observed at 0°C and 30°C. The effect of these adenine nucleotides was tested on mitochondria isolated from duodenum mucosal cells of both rachitic and vitamin D₃-replete chicks, that had been $^{45}\text{Ca}^{2+}$ loaded in vivo. Under the condition used, 90% or more of the $^{45}\text{Ca}^{2+}$ remained in the mitochondria.

Part 1.25.1.2 The effect of parathyroid hormone on release of calcium from mitochondriaMETHOD

The same procedure was followed as described for adenine nucleotides (5.1.1). The mitochondrial suspension containing 4.1 mg protein/ml PTH (highly purified, 2,000 units/mg, a gift from Dr. J.D. Sallis) was added to the succinate medium in place of the adenine nucleotides at a concentration of 20 µg/ml.

RESULTS

Parathyroid hormone had no effect on either rachitic or vitamin D₃-replete chick mitochondria for the first 30 min at 30°C, but the vitamin D₃-replete mitochondria suddenly released $^{45}\text{Ca}^{2+}$ during the period between 30 to 40 min. The results are shown in fig. 6 and are similar to those reported by DeLuca, Engstrom and Rasmussen (1962) for kidney mitochondria.

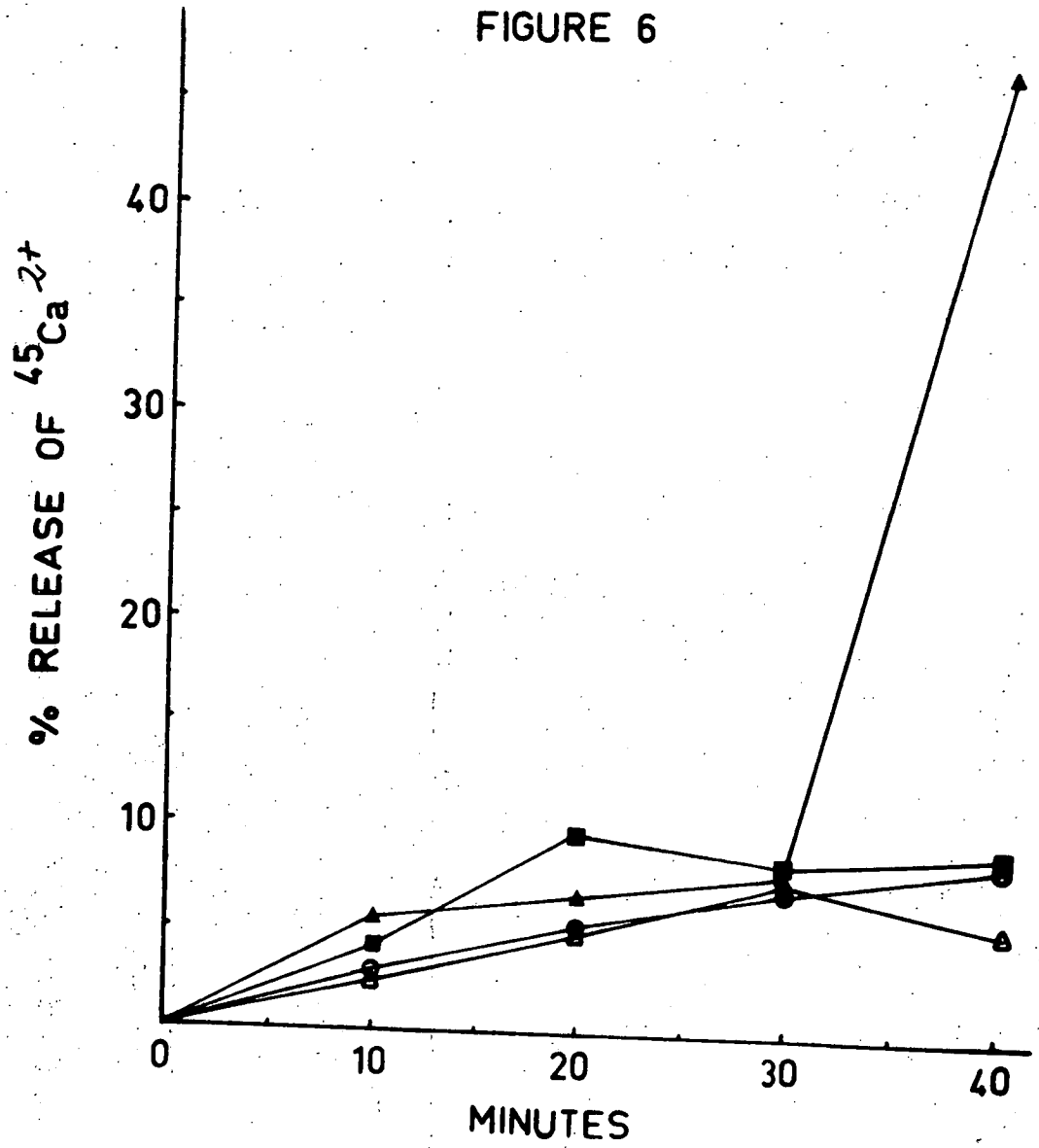
FIGURE 6

The effect of parathyroid hormone on the release of calcium from mitochondria at 30°C

Mitochondria from starved, $^{45}\text{Ca}^{2+}$ loaded, rachitic and vitamin D₃ chick duodenum mucosal cells were prepared and incubated at 30°C as described in 1.5.3.3 and 1.6.3. The percentage of the original $^{45}\text{Ca}^{2+}$ released is plotted against time.

- ——— ○ control mitochondria from rachitic chicks
- Δ ——— Δ mitochondria from rachitic chicks +
parathyroid hormone
- ——— ■ control mitochondria from vitamin D-treated
chicks
- ▲ ——— ▲ mitochondria from vitamin D-treated chicks
+ parathyroid hormone

FIGURE 6



Part 1.35.1.3 The effect of prostaglandins on release of calcium from mitochondriaMETHOD

The mitochondrial suspension was prepared as described in 1.5.3.3 and adjusted to a protein content of 3.5 mg/ml in 0.25M-sucrose + 50mM-Tris Tricine pH 7.1. To 1 ml of the suspension was added 1 ml 0.25M-sucrose + 50mM-Tris Tricine + 10 mg BSA with or without prostaglandin E_1 . These mixtures were incubated at 30°C for 15 min. 500 μ l samples of the mixtures were filtered through millipore membranes as described in 1.6.2.2. The filtrates and the membranes were counted and the results expressed as average of duplicate determinations as % release of total $^{45}\text{Ca}^{2+}$.

RESULTS

Prostaglandin E_1 was found to have no effect on $^{45}\text{Ca}^{2+}$ release from mitochondria at 30°C under similar conditions to those used to study the effect of PTH and adenine nucleotides (Table 45).

TABLE 45

The effect of prostaglandin E_1 on the release of calcium from mitochondria at 30°C

Mitochondria were isolated from starved, $^{45}\text{Ca}^{2+}$ loaded, rachitic chick duodenum mucosal cells and adjusted to 4.5 mg protein/ml in 0.25M-sucrose + 50mM-Tris Tricine + pH 7.1. (1.5.3.3).

To 1 ml mitochondrial suspension was added 1 ml 0.25M-sucrose + 50mM-Tris Tricine pH 7.1 + 10 mg BSA with or without Prostaglandin E_1 . 500 μl sample from the mixture was filtered through millipore filter (1.6.2.2). The filtrates and the membranes were counted. The results are expressed as the percentage release of $^{45}\text{Ca}^{2+}$ from mitochondria.

Final conc. of Prostaglandin M	% release of $^{45}\text{Ca}^{2+}$
none	2.37
10^{-7}	3.56
10^{-6}	2.86
10^{-5}	5.06

Part 1.45.1.4 The effect of EDTA on release of calcium from mitochondriaMETHOD

The mitochondrial suspensions were prepared as described in 1.5.3.3, and adjusted to contain 3.7 mg protein/ml for vitamin D₃-replete chicks and 3.5 mg protein/ml for rachitic chicks in 0.25M-sucrose. 1 ml of the suspensions were added to 1 ml 0.25M-sucrose with or without 2×10^{-4} M-EDTA. The mixtures were incubated at 0°C and 30°C for 15 min. 500 µl samples were taken at 5 min intervals from the mixtures and filtered through millipore membranes as described in 1.6.2.2. The filtrates and residues were counted and the results expressed as average of duplicate determinations as % release of total $^{45}\text{Ca}^{2+}$.

RESULTS

EDTA caused no more release of $^{45}\text{Ca}^{2+}$ from mitochondria at 0°C than released by controls without EDTA. At 30°C it caused release of $^{45}\text{Ca}^{2+}$ from both rachitic and vitamin D₃-replete mitochondria (fig. 7), and the release was linear with time for the 15 min period.

FIGURE 7

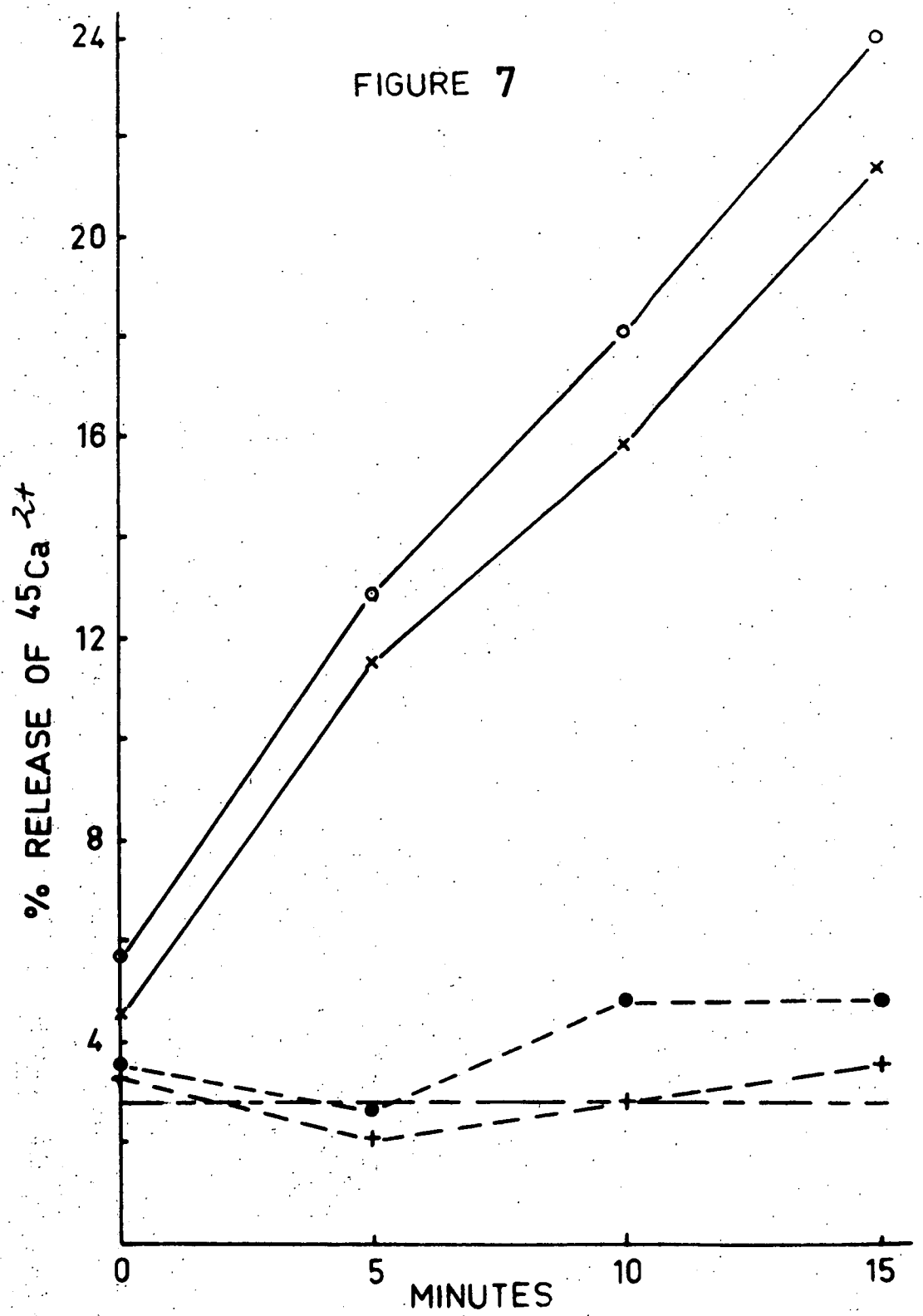
The effect of EDTA on release of calcium from mitochondria at 30°C and 0°C

Mitochondria were isolated from starved, $^{45}\text{Ca}^{2+}$ loaded, rachitic and vitamin D_3 -replete chick duodenum mucosal cells and adjusted to 3.5 mg protein/ml rachitic and 3.7 mg protein/ml vitamin D_3 in 0.25M-sucrose + 50mM-Tris Tricine pH 7.1 (1.5.3.3).

1 ml mitochondrial suspension was added to 1 ml 0.25M-sucrose + 50mM-Tris Tricine pH 7.1 + 10 mg BSA + 10^{-3}M -EDTA. The mixture was incubated for 15 min at 0°C and 30°C (1.6.3). 500 μl sample of the mixture was filtered through millipore filter (1.6.2.2). The filtrate and the membrane were counted. The results are expressed as percent release of $^{45}\text{Ca}^{2+}$ from mitochondria and are the average of duplicate determinations. The percent release was plotted against time.

O ———— O	Vitamin D_3 mitochondria + EDTA at 30°C
X ———— X	rachitic mitochondria + EDTA at 30°C
+ - - - +	vitamin D_3 mitochondria control at 30°C
● - - - ●	rachitic mitochondria control at 30°C
— - - —	control and EDTA vitamin D_3 and rachitic mitochondria at 0°C

FIGURE 7



Part 1.55.1.5 The effect of time on release of calcium from mitochondria due to the presence of crude extracts of calcium binding protein from vitamin D₃-replete and rachitic chicksMETHOD

The mitochondrial suspension was prepared as described in 1.5.3.3 and adjusted to contain 3.5 mg protein/ml. The calcium binding protein extracts were prepared as described in 1.5.4.1. 1 ml mitochondrial suspension was added to 500 μ l 0.5M-sucrose containing 5 mg BSA + 500 μ l Wasserman's Tris buffer pH 7.4 or 500 μ l 0.5M-sucrose + 250 μ l CaBP extract in Wasserman's Tris buffer pH 7.4 + 250 μ l Wasserman's Tris buffer pH 7.4. The mixtures were incubated at 0°C and 30°C for 15 min, with samples being taken at 5 min intervals. The samples were treated and the results expressed as described in 5.1.4.

RESULTS

From fig.8 it can be seen that CaBP extracts incubated at 0°C have no effect on $^{45}\text{Ca}^{2+}$ release over the period of time zero to 15 min. However at 30°C the CaBP extract from vitamin D₃-replete chicks (DCaBP) does exhibit releasing properties while the CaBP extract from rachitic chicks (RCaBP) does not. The release studied over 15 min at 30°C showed that DCaBP will release up to 20% $^{45}\text{Ca}^{2+}$ from these mitochondria. The release of $^{45}\text{Ca}^{2+}$ by DCaBP extract increases linearly with time.

FIGURE 8

The effect of crude calcium binding protein extracts from vitamin D₃-replete and rachitic chick on release of calcium from mitochondria at 0°C and 30°C

Mitochondria were isolated from starved, $^{45}\text{Ca}^{2+}$ loaded, rachitic duodenum mucosal cells and adjusted to 3.5 mg protein/ml in 0.25M-sucrose (1.5.3.3).

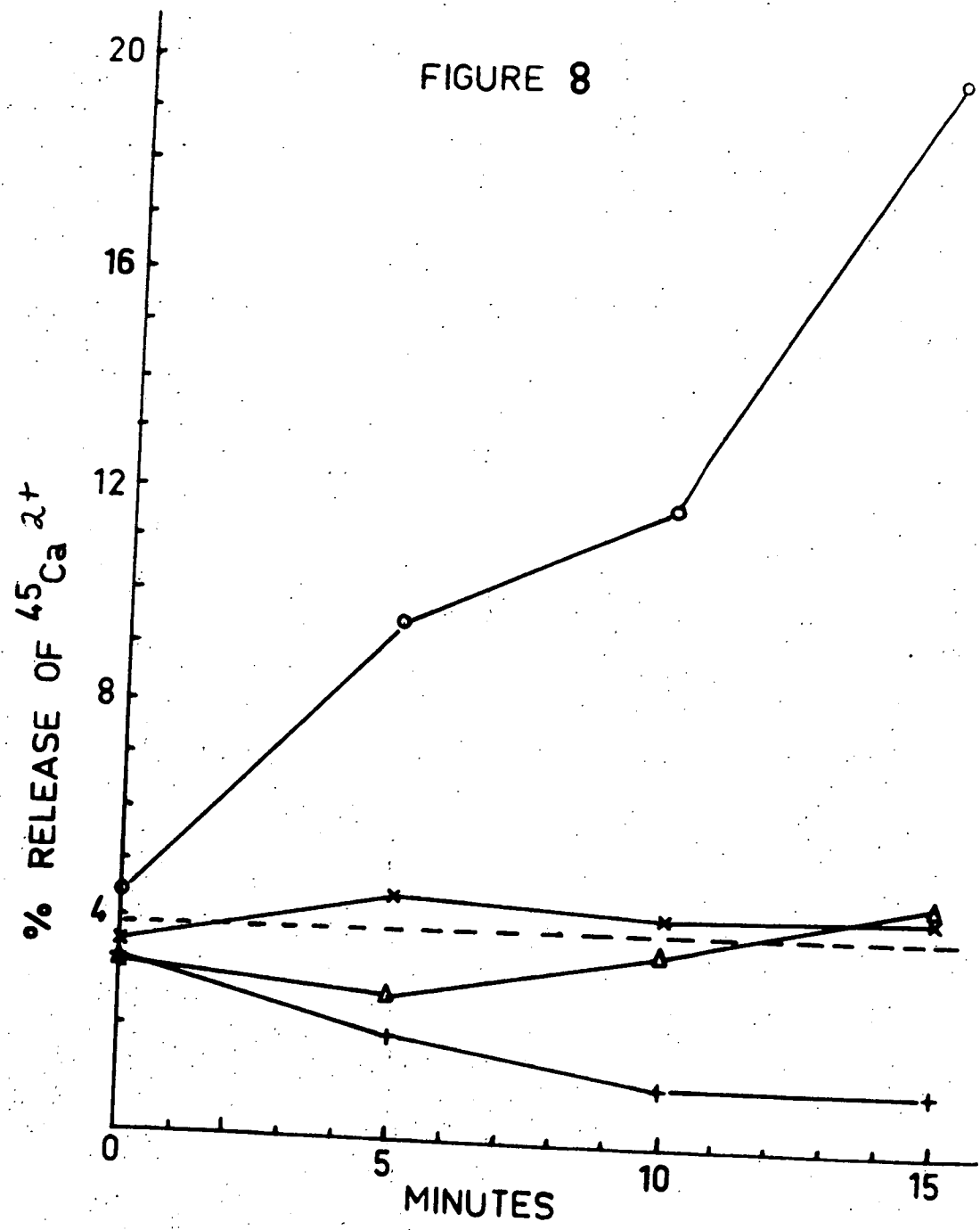
1 ml mitochondrial suspension was added to (1) 500 μl 0.5M-sucrose + 5 mg-BSA + 500 μl Wasserman's Tris buffer pH 7.3 and (2) 500 μl 0.5M-sucrose + 250 μl CaBP extract in Wasserman's Tris buffer + 250 μl Wasserman's Tris buffer pH 7.3.

The CaBP extracts from vitamin D₃-treated chicks contained 10.2 mg protein/ml and 10.5 μg Ca^{2+} /ml, and from rachitic chicks contained 14.2 mg protein/ml and 2.9 μg Ca^{2+} /ml. The extracts were able to bind 31.2 nmoles Ca^{2+} /250 μl for vitamin D₃ and rachitic 4.08 nmoles Ca^{2+} /250 μl at 10^{-4}M Ca^{2+} .

The mixtures were incubated for 15 min at 0°C and 30°C. 500 μl samples were taken and filtered through millipore filter (1.6.2.2). The filtrates and membranes were counted. The results are the average of 9 experiments with duplicate determinations and are expressed as percent release of $^{45}\text{Ca}^{2+}$ from the mitochondria. The percent release of $^{45}\text{Ca}^{2+}$ is plotted against time.

O ————— O	crude vitamin D ₃ CaBP at 30°C
X ————— X	crude vitamin D ₃ CaBP at 0°C
Δ ————— Δ	crude rachitic CaBP at 30°C
+ ————— +	crude rachitic CaBP at 0°C
- - - - -	controls at 0°C and 30°C

FIGURE 8



Part 1.65.1.6 The effect of calcium binding protein extracts on release of calcium from mitochondria from rachitic and vitamin D₃-replete chicksMETHOD

The mitochondrial suspensions were prepared as described in 1.5.3.3 and were adjusted to contain 3.5 mg protein/ml for vitamin D₃-replete and 3.6 mg protein/ml for rachitic chicks in 0.25M-sucrose + 50mM-Tris Tricine pH 7.1. The CaBP extracts were prepared as described in 1.5.4.1. The mixtures were set up as described in 1.6.3 and incubated at 0°C and 30°C for 15 min. The method of sampling and treatment of the results are described in 5.1.5.

RESULTS

Mitochondria from either rachitic or vitamin D₃-replete chicks did not release their $^{45}\text{Ca}^{2+}$ with either of the CaBP extracts at 0°C. At 30°C the CaBP extract from vitamin D-replete chicks caused a similar release of $^{45}\text{Ca}^{2+}$ from both rachitic and vitamin D₃-replete chick mitochondria. The CaBP extract from rachitic chick caused no more release of $^{45}\text{Ca}^{2+}$ than did the control mitochondria. Again with CaBP from vitamin D₃-replete chick the release was linear with time (fig.9).

FIGURE 9

The effect of crude calcium binding protein extracts on release of calcium from mitochondria of rachitic and vitamin D₃ replete chicks at 0°C and 30°C

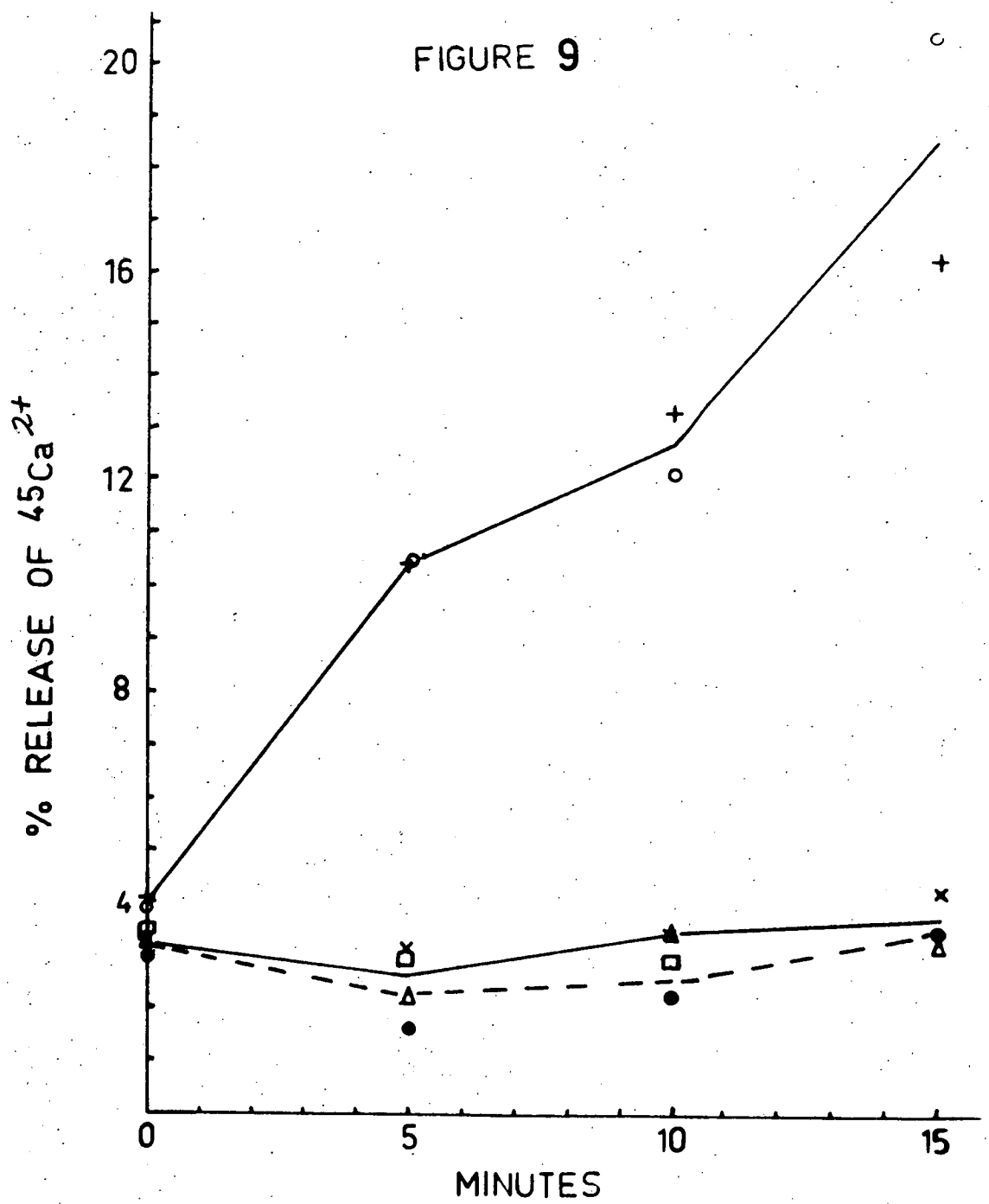
Mitochondria were isolated from starved, ⁴⁵Ca²⁺ loaded, vitamin D₃-replete and rachitic duodenum mucosal cells and adjusted to 3.5 mg protein/ml for vitamin D₃ and 3.6 mg protein/ml for rachitic in 0.25M-sucrose (1.5.3.3).

1 ml mitochondrial suspension was added to (1) 500 µl 0.5M-sucrose + 5 mg BSA + 500 µl Wasserman's Tris buffer pH 7.3 and (2) 500 µl 0.5M-sucrose + 250 µl CaBP extract in Wasserman's Tris buffer pH 7.3 + 250 µl Wasserman's Tris buffer pH 7.3.

250 µl CaBP extracts from vitamin D₃-treated chicks were able to bind vitamin D₃ 31.2 nmoles Ca²⁺ and from rachitic chicks 4.08 nmoles Ca²⁺ at 10⁻⁴M Ca²⁺. The mixtures were incubated for 15 min at 0°C and 30°C. 500 µl samples were taken at time zero, 5, 10 and 15 min and filtered through millipore membrane (1.6.2.2). The filtrates and the membranes were counted. The results are the average of 3 experiments with duplicates and are expressed as percent release of ⁴⁵Ca²⁺ from mitochondria. The percent of ⁴⁵Ca²⁺ release is plotted against time.

○ ————— ○	vitamin D ₃ mitochondria + crude D ₃ CaBP
+ ————— +	rachitic mitochondria + crude D ₃ CaBP
X ————— X	vitamin D ₃ mitochondria + crude R CaBP
Δ ————— Δ	rachitic mitochondria + crude R CaBP
● ————— ●	vitamin D ₃ mitochondria control
□ ————— □	rachitic mitochondria control

FIGURE 9



Part 1.75.1.7 The effect of calcium binding protein concentration on release of calcium from mitochondriaMETHOD

The isolation of the mitochondria and the incubation procedure used is described in 5.1.6. The mitochondrial suspension contained 4.4 mg/ml. The CaBP extracts were prepared as described in 1.5.4.1. The results are expressed as % release of total $^{45}\text{Ca}^{2+}$.

RESULTS

From Table 46, one can see that increasing the amount crude extract of CaBP from vitamin D_3 -replete chick increased the release of $^{45}\text{Ca}^{2+}$ from mitochondria prepared from vitamin D_3 -replete chick. However this was not observed for the crude CaBP extract from rachitic chicks, which shows only 6% at the highest level used.

TABLE 46

The effect of concentration of crude calcium binding protein on calcium release from mitochondria at 30°C

Mitochondria were isolated from starved, $^{45}\text{Ca}^{2+}$ loaded, vitamin D_3 -replete chick duodenum mucosal cells and adjusted to 4.4 mg protein/ml in 0.25M-sucrose (1.5.3.3).

1 ml mitochondrial suspension was added to (1) 500 μl 0.5M-sucrose + 5 mg BSA + 600 μl Wasserman's Tris buffer pH 7.3 and (2) 500 μl 0.5M-sucrose + 150-600 μl CaBP extract in Wasserman's Tris buffer pH 7.3 + 450-0 μl Wasserman's Tris buffer pH 7.3.

The crude CaBP extract from vitamin D_3 -treated chicks contained 10.2 mg protein/ml and 10.5 μg Ca^{2+} /ml and from rachitic chicks contained 14.2 mg protein/ml and 2.9 μg Ca^{2+} /ml. 250 μl CaBP extract were able to bind vitamin D_3 31.2 nmoles Ca^{2+} and rachitic 4.08 nmoles Ca^{2+} as tested at 10^{-4}M Ca^{2+} .

The mixtures were incubated for 15 min at 30°C. 500 μl samples were taken at time 15 min and filtered through millipore membrane (1.6.2.2). The filtrate and membranes were counted. The results are the average of duplicate determinations and are expressed as percent of $^{45}\text{Ca}^{2+}$ released from mitochondria.

Amount of crude CaBP mg	Type of CaBP	% release of $^{45}\text{Ca}^{2+}$
<u>Vitamin</u>		
none	D_3	2.9
1.5	D_3	5.4
3.0	D_3	32.0
6.0	D_3	64.0
<u>Rachitic</u>		
none	R	3.0
2.0	R	3.7
4.0	R	4.1
8.0	R	5.5

Part 1.85.1.8 The fractionation of calcium binding protein extracts by Diaflo membranes and the effect of the fractions on calcium release from mitochondriaMETHOD

A vitamin D₃-replete chick crude extract of CaBP prepared as described in 1.5.4.1 was sorted out according to molecular size by passing through successive XM 100, XM 50, PM 30 and UM 10 Diaflo membranes. The residues that remained were made up to the original volume of the extract. These residues were tested for their release of $^{45}\text{Ca}^{2+}$ from rachitic chick mitochondria. The mitochondria were isolated and the incubation done as described in 5.1.6. The mitochondrial suspension contained 3.8 mg protein/ml. The results are expressed as % release of the total $^{45}\text{Ca}^{2+}$.

RESULTS

After passing the crude CaBP extract through Diaflo membranes (Amicon Corp., U.S.A.), the capacity to binding $^{45}\text{Ca}^{2+}$, as tested by the chelex method, was located mainly in the material retained by the XM 50 membrane as shown in Table 47. The ability of the fraction to release $^{45}\text{Ca}^{2+}$ from loaded mitochondria was also located in the same fraction (Table 47). Again the amount of $^{45}\text{Ca}^{2+}$ released was proportional to the amount of extract added.

TABLE 47

The effect of crude calcium binding protein and ultrafiltrates residues on calcium release from mitochondria at 0°C and 30°C

Mitochondria from starved, $^{45}\text{Ca}^{2+}$ loaded rachitic duodenum mucosal cells were isolated and adjusted to 3.8 mg protein/ml in 0.25M-sucrose (1.5.3.3).

1 ml mitochondrial suspension was added to (1) 500 μl 0.5M-sucrose + 5 mg-BSA + 500 μl Wasserman's Tris buffer pH 7.3 and (2) 500 μl 0.5M-sucrose + 10-500 μl CaBP extract in Wasserman's Tris buffer + 490-0 μl Wasserman's Tris buffer pH 7.3.

The mixtures were incubated for 15 min at 0°C and 30°C. 500 μl samples were taken after 15 min incubation and filtered through millipore membrane (1.6.2.2). The filtrates and membranes were counted. The results are the average of duplicate determinations and are expressed as the percentage release of $^{45}\text{Ca}^{2+}$ from mitochondria.

The binding activity of the crude CaBP and the residues were tested at 10^{-5}M Ca^{2+} .

The Diaflo residues remained after passing the crude CaBP through these membranes.

TABLE 47

Extracts	Volume μl	Protein mg/vol. added	$^{40}\text{Ca}^{2+}$ bound nmoles/vol.	% release of $^{45}\text{Ca}^{2+}$ from mitochondria	
				0°C	30°C
none	0	0.00	0.00	3.5	5.0
crude CaBP	250	11.20	8.70	6.9	34.6
XM 100	100	3.87	3.41	11.0	71.1
XM 100	50	1.94	1.70	-	55.4
XM 100	25	0.97	0.85	-	16.1
XM 100	10	0.39	0.34	-	6.9
XM 50	150	3.54	3.90	13.2	72.6
XM 50	100	2.36	2.62	-	60.0
XM 50	25	0.59	0.65	-	9.4
PM 30	250	2.78	1.95	5.8	54.8
UM 10	500	3.80	1.06	4.1	53.2

- not determined

Part 1.95.1.9. The effect of pure calcium binding protein on calcium release from mitochondriaMETHOD

The mitochondrial suspensions were prepared and incubated as described in 5.1.6. The mitochondria suspensions were adjusted to contain 3.4 mg protein/ml for vitamin D₃-replete and 3.5 mg/protein/ml for rachitic chicks. The pure CaBP preparations tested were gifts from Professors R.H. Wasserman and E.S. Holdsworth. The results are expressed as % release of total $^{45}\text{Ca}^{2+}$.

RESULTS

To be sure that the effects observed were due to the CaBP of Wasserman, a sample of the pure material (generously supplied by Prof. R.H. Wasserman) was tested in the usual system, and caused release of $^{45}\text{Ca}^{2+}$ from loaded mitochondria only at 30°C (fig.10). The small amount of material available precluded its use at more than one concentration. The percent of $^{45}\text{Ca}^{2+}$ released was only 9% of the total $^{45}\text{Ca}^{2+}$ and it was considered that the protein may have deteriorated in the passage to Tasmania. Accordingly, similar material was prepared in this laboratory (a gift from Prof. E.S. Holdsworth) and tested at several different concentrations as shown in fig. 11. The release of $^{45}\text{Ca}^{2+}$ by pure CaBP increased with increase in concentrations and was linear with time.

FIGURE 10

The effect of pure calcium binding protein on calcium release from mitochondria at 0°C and 30°C

Mitochondria from starved, $^{45}\text{Ca}^{2+}$ loaded, rachitic and vitamin D_3 -replete chick duodenum mucosal cells were isolated and adjudged to 3.4 mg protein/ml vitamin D_3 and 3.5 mg protein/ml for rachitic in 0.25M-sucrose (1.5.3.3).

1 ml mitochondrial suspension was added to (1) 500 μl 0.5M-sucrose + 5 mg-BSA + 500 μl Wasserman's Tris buffer pH 7.3 and (2) 500 μl 0.5M-sucrose + 100 μl pure CaBP (prepared by Wasserman) + 400 μl Wasserman's Tris buffer pH 7.3.

The mixtures were incubated for 15 min at 0°C and 30°C. 500 μl samples were taken at times zero, 5, 10 and 15 min and filtered through millipore membranes (1.6.2.2). The filtrates and the membranes were counted. The results are the average for duplicate determinations of 2 experiments. The percentage release of $^{45}\text{Ca}^{2+}$ from mitochondria is plotted against time.

0 ————— 0	pure CaBP 100 μg (prepared by Wasserman) at 30°C for rachitic and vitamin D_3 mitochondria
+ ----- +	pure CaBP 100 μg at 0°C
X ————— X	control at 30°C
Δ ----- Δ	control at 0°C

FIGURE 10

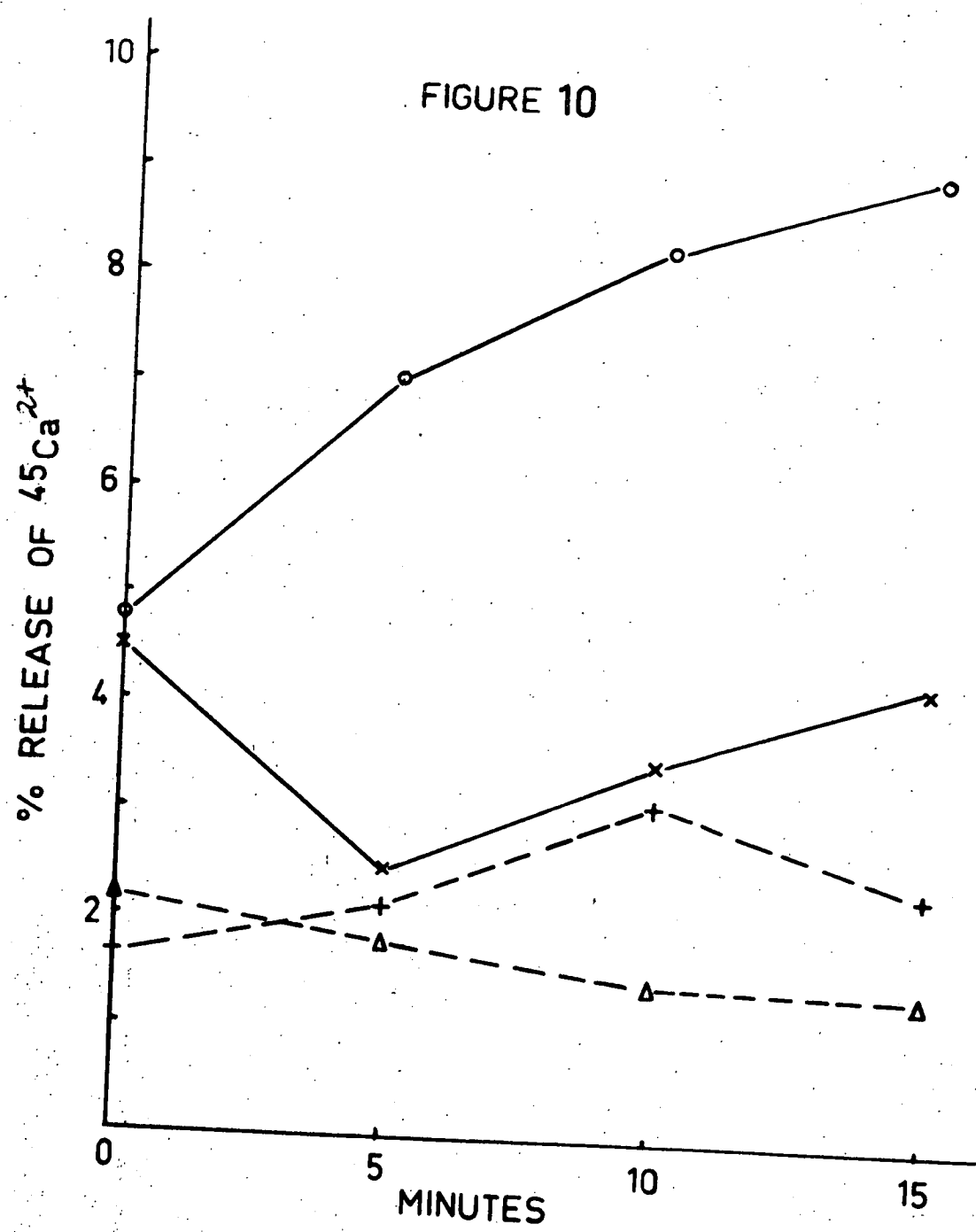


FIGURE 11

The effect of concentration of pure calcium binding protein on calcium release from mitochondria at 30°C

Mitochondria from starved, $^{45}\text{Ca}^{2+}$ loaded, rachitic chick duodenum mucosal cells were isolated and adjusted to 3.9 mg protein/ml in 0.25M-sucrose + 50mM-Tris Tricine pH 7.1 (1.5.3.3).

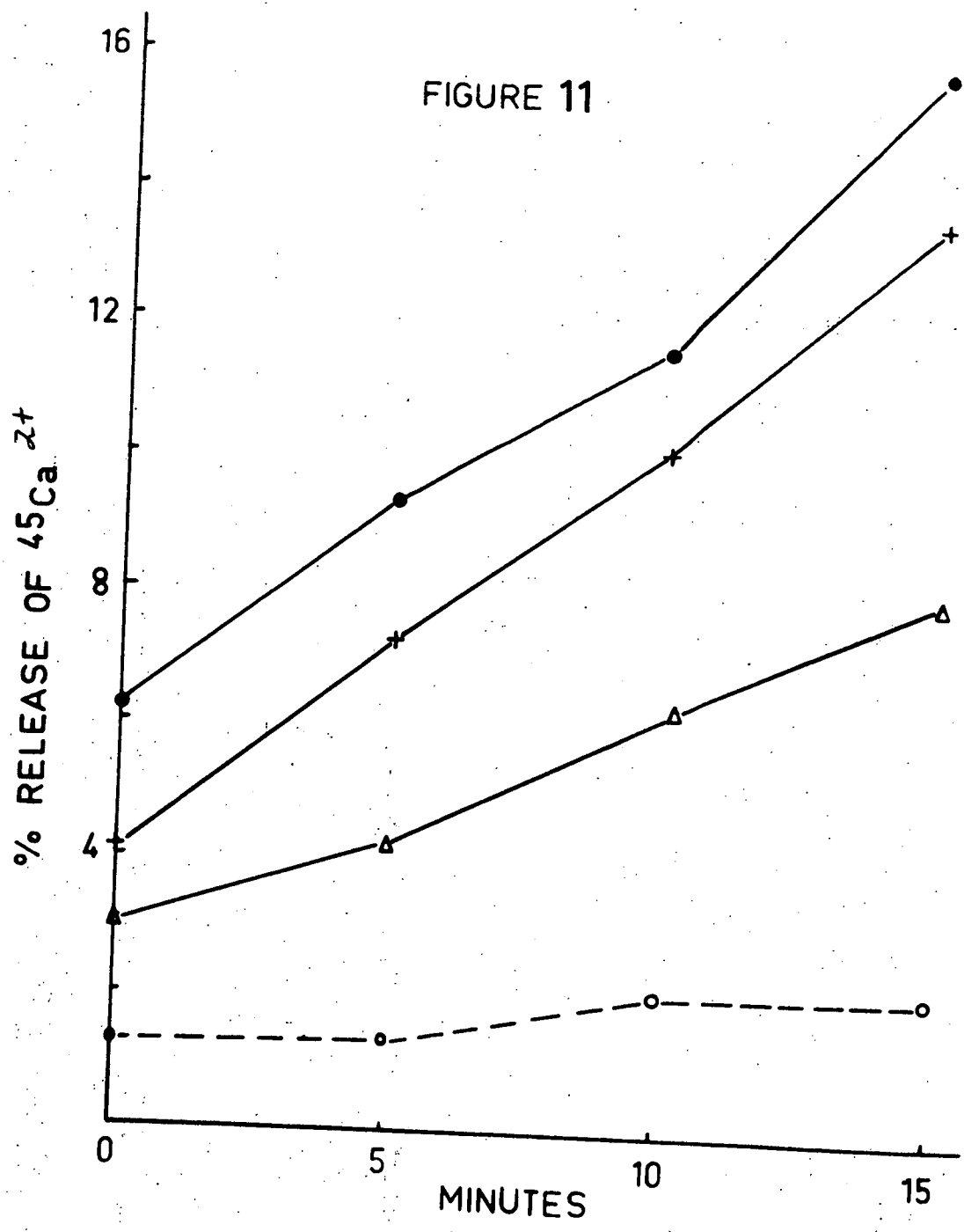
1 ml mitochondrial suspension was added to (1) 500 μl 0.5M-sucrose + 5 mg BSA + 500 μl Wasserman's Tris buffer pH 7.3 and (2) 500 μl 0.5M-sucrose + 5 mg BSA + 25-100 μl pure CaBP + 475-400 μl Wasserman's Tris buffer pH 7.3.

The pure CaBP was able to bind 3 nmoles Ca^{2+} /210 μg protein as tested at 10^{-5}M Ca^{2+} .

The mixtures were incubated for 15 min at 30°C. 500 μl samples were taken after 15 min and filtered through millipore membrane (1.6.2.2). The filtrates and the membranes were counted. The results are the average of duplicate determinations and are expressed as percentage $^{45}\text{Ca}^{2+}$ released from mitochondria. The percentage release is plotted against time

● ————— ●	pure CaBP 210 μg (prepared by Holdsworth)
+ ————— +	pure CaBP 105 μg
Δ ————— Δ	pure CaBP 53 μg
○ - - - - - ○	control

FIGURE 11



PART 25.2 The effect of calcium binding protein on the uptake of calcium by mitochondria

CaBP has been found to be release Ca^{2+} from mitochondria (5.1). It is necessary to know whether CaBP will prevent the accumulation of Ca^{2+} by mitochondria and in this part the effect of crude and pure CaBP preparation on the uptake of $^{45}\text{Ca}^{2+}$ by isolated mitochondria was examined.

Part 2.15.2.1 The effect of crude calcium binding protein on calcium uptake by mitochondriaMETHOD

Mitochondria were prepared as described in 1.5.3.3 and the $^{45}\text{Ca}^{2+}$ uptake studied as described in 1.6.2.2. The results are expressed as a percent $^{45}\text{Ca}^{2+}$ uptake by the mitochondria.

RESULTS

At 0°C , mitochondria in the presence and absence of CaBP extracts from vitamin D-replete chicks and rachitic chicks had taken up 35% of $^{45}\text{Ca}^{2+}$ after 9 min. Thus the binding proteins had no effect on Ca^{2+} uptake at 0°C (fig.12).

FIGURE 12

The effect of crude calcium binding protein extract on calcium uptake by mitochondria

Non-loaded mitochondria were isolated from rachitic chicks prepared in 0.25M-sucrose and was adjusted to 3.5 mg protein/ml

(1.5.3.3). The mitochondria were incubated at 0°C for 9 min

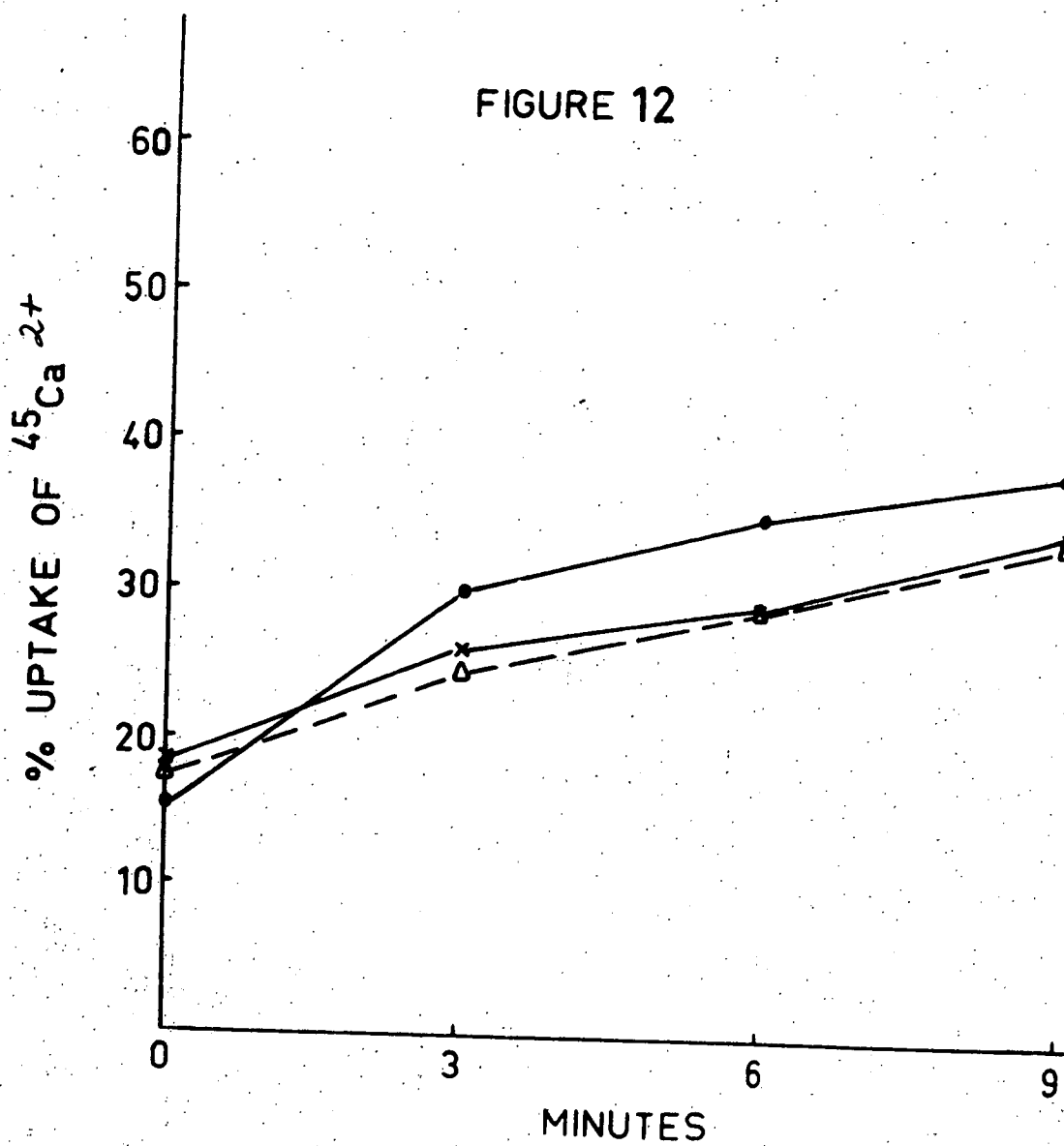
(1.6.2.2).

250 μ l crude CaBP were added to the incubation of 2 ml mitochondrial suspension + 500 μ l 0.164M-sucrose + 62mM-imidazole + 56mM-succinate + 5 mg BSA + 250 μ l 0.25M-sucrose. At time zero, 25 μ l $^{45}\text{Ca}^{2+}/^{40}\text{Ca}^{2+}$ solution (10 μ g, 52,000 C.P.M.) were added to the mixture.

The results are the average of 2 experiments. The crude CaBP preparation 284 nmoles/250 μ l for vitamin D₃ extract and 10 nmoles/250 μ l for rachitic extract as tested at 10^{-4} M Ca^{2+} .

● ————— ●	control 0°C
X ————— X	DCaBP 0°C
Δ - - - - - Δ	RCaBP 0°C

FIGURE 12



Part 2.25.2.2 The effect of calcium binding protein Diaflo extract on calcium uptake by mitochondriaMETHOD

Mitochondria were prepared as described in 1.5.3.3 and the $^{45}\text{Ca}^{2+}$ uptake studied as described in 1.6.2.2. The results are expressed as a percent $^{45}\text{Ca}^{2+}$ uptake by the mitochondria.

RESULTS

In Table 48 on the effect of Diaflo residues on Ca^{2+} uptake by mitochondria, one can see there is little or no inhibition of uptake at 0°C except the XM 50 residue, which shows 14% difference from the control. This is similar to the effect of this residue on release. While at 30°C , the difference from control after 15 min was maximal with 100 μl XM 100 at 70%, and decreased from there to PM 30 at 46%.

TABLE 48

The effect of calcium binding protein diaflo residues on calcium uptake by mitochondria

Mitochondria were isolated from starved rachitic chick duodenum mucosal cells as described in 1.5.3.3. The suspension contained 3.2 mg protein/ml. 2 ml mitochondrial suspension in 0.25M-sucrose was added to 500 μ l 0.164M-sucrose + 62mM-imidazole + 56mM-succinate + 5 mg BSA with or without 100-500 μ l CaBP extract residue (Diaflo) in Tris buffer (Wasserman and Taylor, 1966) with or without 400-0 μ l 0.25M-sucrose. The mitochondria were preincubated for 5 min with these residues before the addition of 25 μ l $^{45}\text{Ca}^{2+}/^{40}\text{Ca}^{2+}$ solution (7.5 μ g, 0.5 μ Ci). A sample was removed from the incubation mixture after 15 min and was filtered through millipore filters as described 1.6.2.2. The results are calculated as a percent of the total $^{45}\text{Ca}^{2+}$ added and are the average of duplicate determinations.

Residue of Diaflo	Volume of residue μ l	Ca^{2+} bound nmoles /vol.	% uptake 0°C	% inhibit- ion of uptake	% uptake 30°C	% inhibit- ion of uptake
none	-	-	74	0	88	0
crude CaBP	250	8.70	73	1	27	61
XM100	100	3.41	75	*1	18	70
XM50	150	3.90	60	14	28	60
PM30	250	1.95	70	4	42	46
UM10	500	1.06	79	*5	31	57

* uptake greater than control

Part 2.35.2.3 The effect of pure calcium binding protein on calcium on uptake by mitochondriaMETHOD

Mitochondria were prepared as described in 1.5.3.3 and the $^{45}\text{Ca}^{2+}$ uptake studies as described in 1.6.2.2. The results are expressed as a percent $^{45}\text{Ca}^{2+}$ uptake by the mitochondria.

RESULTS

In fig.13 it appeared that pure CaBP (gift from Prof. E.S. Holdsworth) caused a decreased uptake of $^{45}\text{Ca}^{2+}$ by mitochondria at 30°C. The method used could not distinguish between prevention of uptake or an effect of CaBP on efflux of $^{45}\text{Ca}^{2+}$ previously taken up in the first 20 s required to perform the addition, mixing and filtration of the $^{45}\text{Ca}^{2+}$ with the mitochondria. The amount of $^{45}\text{Ca}^{2+}$ used could be taken up rapidly. The absence of substrate from the incubation mixture would account for the release of $^{45}\text{Ca}^{2+}$ from the control mitochondria. However the presence of increasing amounts of CaBP led to less $^{45}\text{Ca}^{2+}$ remaining in the mitochondria after 15 min.

FIGURE 13

The effect of pure calcium binding protein on calcium uptake by mitochondria at 30°C

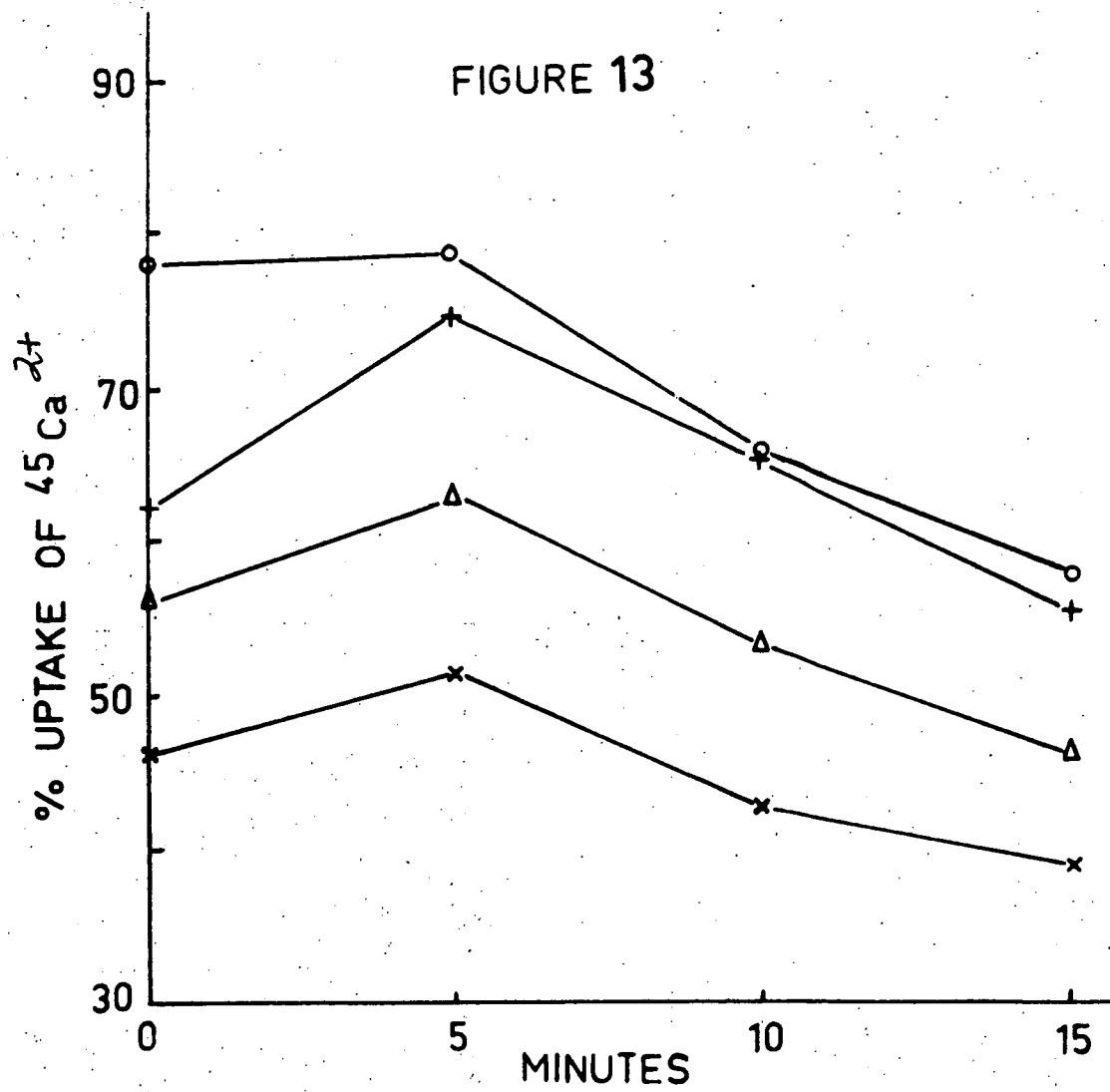
Non-loaded mitochondria were isolated from starved, rachitic chicks duodenum mucosal cells and adjusted to 6.0 mg protein/ml in 0.25M-sucrose + 50mM-Tris Tricine pH 7.2 (1.5.3.3). 1 ml mitochondrial suspension was added to 100 to 25 μ l pure CaBP (Holdsworth) + 400-475 μ l 0.25M-sucrose + 50mM-Tris Tricine pH 7.5 + 4.5 mg BSA. The mitochondria were incubated at 30°C for 15 min after the addition of 25 μ l $^{45}\text{Ca}^{2+}/^{40}\text{Ca}^{2+}$ solution (7.5 μ g, 0.5 uCi). Samples were taken at zero, 5, 10 and 15 min and filtered through millipore filter as described in 1.6.3.3. The filtrate and membrane were counted. The percent of the total $^{45}\text{Ca}^{2+}$ taken up was calculated. The results are the average of duplicate determinations.

294 μ g pure CaBP (Holdsworth) was able to bind 4.2 nmoles Ca^{2+} at final Ca^{2+} concentration of 10^{-5}M .

The percentage of $^{45}\text{Ca}^{2+}$ taken up is plotted against time.

0 ————— 0	control
+ ————— +	74 μ g pure CaBP
Δ ————— Δ	147 μ g pure CaBP
X ————— X	294 μ g pure CaBP

FIGURE 13



Discussion

In this chapter an attempt was made to find a substance likely to be present in the cell which could cause the release of Ca^{2+} from mitochondria. None of the adenine nucleotides, including the artificial dibutyryl cyclic AMP, caused Ca^{2+} release. It is known that DBCAMP is able to penetrate membranes. Thus, the lack of an effect by these adenine nucleotides is not because of this problem. Prostaglandin E_1 also lacked an effect on Ca^{2+} release from mitochondria. Previous work from DeLuca's laboratory suggested that PTH might be the physiological substance sought, since this hormone caused release of Ca^{2+} from kidney mitochondria of vitamin D-replete but not rachitic rats (DeLuca, Engstrom and Rasmussen, 1962). The release of Ca^{2+} did not occur earlier than 20 min from the start of incubation. Essentially, similar results were obtained in the work reported here using mitochondria loaded with $^{45}\text{Ca}^{2+}$ in vivo, prepared from duodena of vitamin D_3 -replete chicks (fig.6). No release of $^{45}\text{Ca}^{2+}$ from the rachitic mitochondria occurred even on incubation for times up to 40 min. Thus the synergistic effect of vitamin D and PTH might cause the release of Ca^{2+} from mitochondria, but the effect was not observed until 30 min incubation, then the release was abrupt and may have been due to a sudden loss of integrity of the mitochondria membrane structures.

The CaBP either in crude form or as the pure protein did cause the release of $^{45}\text{Ca}^{2+}$ from intestinal mitochondria preloaded with $^{45}\text{Ca}^{2+}$. The release was not produced at 0°C but at 30°C and increased linearly with time and in proportion to the amount of

CaBP present. The release of $^{45}\text{Ca}^{2+}$ was obtained with mitochondria isolated from either rachitic or vitamin D-replete chicks. Thus the absence of CaBP as in the rachitic chick may be the reason why the mitochondria of rachitic chicks do not turnover their Ca^{2+} as rapidly as vitamin D-replete chicks.

The CaBP either crude or in pure form did not interfere with $^{45}\text{Ca}^{2+}$ uptake by mitochondria at 0°C , but did result in a lower accumulation of $^{45}\text{Ca}^{2+}$ in mitochondria at 30°C , although it was difficult to differentiate between prevention of uptake or rapid uptake of Ca^{2+} followed by its release (fig. 13).

The chelating agent EDTA mimicked the effect of CaBP in that it would cause the release of Ca^{2+} from mitochondria at 30°C but not at 0°C . The effective concentrations used were vastly different, eg. EDTA was at 10^{-4}M and CaBP at $7 \times 10^{-9}\text{M}$. Another difference observed was that EDTA would prevent $^{45}\text{Ca}^{2+}$ uptake by mitochondria at 0°C whereas CaBP had little or no effect at this temperature.

CHAPTER 6EXAMINATION OF CALCIUM TRANSPORT BY THE SMALL INTESTINEIntroduction

Previous work in thesis has been primarily concerned with intracellular events. Vitamin D has been postulated to enhance the permeability of the mucosal surface by some unknown process or possibly by the presence of the CaBP as a carrier in the surface. By placing excess CaBP in the lumen, an attempt was made to increase translocation of Ca^{2+} in rachitic chicks.

Work by Webling and Holdsworth had shown that certain bile salts increased the permeability of the mucosal surface to Ca^{2+} (Webling and Holdsworth 1965; 1966). Thus the effect of sodium taurochenodeoxycholate on Ca^{2+} transport in rachitic chicks was studied, to see if increasing the permeability of the cell surface would mimic the effect of vitamin D.

If vitamin D increases the permeability of the mucosal surface this might be a two way process. Therefore the exit of Ca^{2+} from plasma to lumen was studied.

Recently Moore (1971) and Vasington et al (1972) reported that ruthenium red, a mucopolysaccharide stain, inhibited energy dependent uptake of Ca^{2+} by mitochondria. As mucopolysaccharides are located at the microvilli an investigation of its effect on Ca^{2+} translocation was examined.

Calcium accumulated by the intestinal cells may have to be pumped out of the cell to the extracellular fluid. A very recent paper by Birge, Gilbert and Avioli (1972), showed that ethacrynic

acid inhibited Ca^{2+} transport in the everted ileal sac. Therefore the effect of this substance on the in vivo transport of Ca^{2+} was studied.

The effect of temperature upon the Ca^{2+} transport process in the intestine was examined, because Rasmussen et al (1963) had shown a Ca^{2+} uptake by isolated villi and a temperature dependent release. The ileum was used in the study because of the higher Ca^{2+} to wet weight ratio.

PART 16.1 The effect of calcium binding protein on mucosal cell uptake of calciumMETHOD

$^{45}\text{Ca}^{2+}/^{40}\text{Ca}^{2+}$ solutions (40 $\mu\text{g}/\text{ml}$ 11.0 $\mu\text{Ci}/\text{ml}$) with and without CaBP (pure 2 mg/ml) were prepared. Another solution was prepared containing $^{45}\text{Ca}^{2+}/^{40}\text{Ca}^{2+}$ (4 mg/ml , 10 $\mu\text{Ci}/\text{ml}$) with and without 6.6 mg CaBP (pure)/ ml .

500 μl or 1 ml of one of these solutions was placed in the lumen of the duodenum of starved rachitic chicks for 20 to 60 min (1.4.1 and 1.4.2). The mucosal cells were isolated and homogenized in 0.25M-sucrose + 2mM- Mg^{2+} + 50mM-Tris Tricine pH 7.3 at 0°C (1.5.1 and 1.5.2). Blood and bone samples were taken and treated as described in 1.7.3.1 and 1.7.4.2. $^{45}\text{Ca}^{2+}$, $^{40}\text{Ca}^{2+}$ and protein were estimated (1.8).

RESULTS

The presence of CaBP in the intestinal lumen with $^{45}\text{Ca}^{2+}$ has not enhanced Ca^{2+} transfer into the mucosal cell during the 20 min period as shown in Table 49. It is also apparent from the bone samples that CaBP has not increased Ca^{2+} translocation from the cell to plasma for 20 min study period (Table 49). After 1 h, loading of the loop with $^{45}\text{Ca}^{2+}$ plus CaBP has increased the cellular content by about one and a half fold, as compared to $^{45}\text{Ca}^{2+}$ alone. As the cells scraped from the duodenum of chick were bulked, thus individual variations and the significance of the increase in cellular Ca^{2+} could not be tested. However, blood samples taken at 20 min and bone

samples taken at 1 h do not show any difference in the translocation of $^{45}\text{Ca}^{2+}$ to plasma or bone (Table 50).

TABLE 49

The effect of calcium binding protein on calcium transport from the lumen

500 μ l $^{45}\text{Ca}^{2+}/^{40}\text{Ca}^{2+}$ solution (20 μ g, 5.5 μ Ci) with or without CaBP (pure, 2 mg) were placed in the duodenal loops of starved rachitic chicks for 20 min (1.4.1 and 1.4.2). The mucosal cells were isolated after washing the loop externally and internally and then homogenized in 0.25M-sucrose + 2mM-Mg $^{2+}$ + 50mM-Tris Tricine pH 7.3 (1.5.1 and 1.5.2). A bone sample was taken after 20 min and treated as described in 1.7.4.2. The homogenate was estimated for $^{45}\text{Ca}^{2+}$, $^{40}\text{Ca}^{2+}$ and protein (1.8). The results are the average for 4 chicks expressed as per duodenum.

Treatment	MUCOSAL CELLS			TIBIA
	Protein mg	$^{40}\text{Ca}^{2+}$ μ g	C.P.M.	C.P.M./ whole tibia
$^{45}\text{Ca}^{2+}$ only	213	74	2,846,375	14,172 ^x
$^{45}\text{Ca}^{2+}$ + CaBP	228	71	1,933,103	15,399 ^x

level of significance

x 0.8 < P < 0.9

TABLE 50

The effect of calcium binding protein on calcium transport from the lumen

1.0 ml $^{45}\text{Ca}^{2+}/^{40}\text{Ca}^{2+}$ solution (4 mg, 10 μCi) with or without CaBP (pure 2 mg) was placed in the duodenal loop of starved rachitic chicks for 1 h (1.4.1 and 1.4.2). The mucosal cells were isolated after washing the loop externally and internally and then homogenized in 0.25M-sucrose + 2mM-Mg $^{2+}$ + 50mM-Tris Tricine pH 7.1 (1.5.1 and 1.5.2). Blood samples were taken at 20 min and bone samples after 1 h and were treated as described in 1.7.3.1 and 1.7.4.2. The results are the average for 4 chicks expressed as per chick.

MUCOSAL CELLS					
Treatment	Protein mg	$^{40}\text{Ca}^{2+}$ ug	C.P.M.	C.P.M./ml plasma at 20 min	C.P.M./whole tibia at 1 h
$^{45}\text{Ca}^{2+}$ only	178	139	1,595,550	1,609 ⁺	21,710 ^x
$^{45}\text{Ca}^{2+}$ + CaBP	170	201	2,183,499	1,364 ⁺	25,176 ^x

level of significance of the difference between:-

+ 0.3 < P < 0.4

x 0.3 < P < 0.4

PART 26.2 The effect of ruthenium red on calcium transport by the duodenumMETHOD

Duodenal loops of rachitic and vitamin D-replete chicks were loaded with 500 μ l $^{45}\text{Ca}^{2+}/^{40}\text{Ca}^{2+}$ solution (2 mg, 5 μ Ci) with or without 0.7 mg ruthenium red for 25 min (1.4.1 and 1.4.2). The loops were removed and washed internally and externally. The mucosal cells were isolated as described in 1.5.1 and weighed. The isolated cells were digested as described 1.7.5. A bone sample was taken from each bird and treated as described in 1.7.4.1.

RESULTS

Ruthenium red as shown in Table 51 appears to have no influence on Ca^{2+} transport, even though it stained the mucopolysaccharides of the brush borders. The $^{45}\text{Ca}^{2+}$ cellular and bone contents of both rachitic and vitamin D_3 -treated chicks show that ruthenium red has not inhibited the entry or exit of Ca^{2+} into or out of the cell. The usual effect of vitamin D on Ca^{2+} transport is shown by accumulation of $^{45}\text{Ca}^{2+}$ by bone (Table 51).

TABLE 51

The effect of ruthenium red on calcium transport by the duodenum

Duodenal loops of starved, rachitic and vitamin D₃-replete chicks were loaded with 500 μ l $^{45}\text{Ca}^{2+}/^{40}\text{Ca}^{2+}$ solution (2 mg, 5 μ Ci) with or without 0.7 mg ruthenium red for 25 min (1.4.1 and 1.4.2). The loop was removed, washed internally and externally. The mucosal cells were isolated (1.5.2). The cells were weighed and then digested as described in 1.7.5. A bone sample was taken and treated as described in 1.7.4.1. The results are the average for 3 duodena expressed as per duodenum.

Type of chick	ruthenium red	MUCOSAL CELLS	TIBIA
		C.P.M./g wet weight of cells	C.P.M./ashed tibia
Vitamin D ₃ -treated	-	267,949 ^{a,b}	82,290 ^{v,w}
Rachitic	-	296,210 ^{b,c}	14,449 ^{v,x}
Vitamin D ₃ -treated	+	184,274 ^{a,d}	68,230 ^{y,w}
Rachitic	+	336,534 ^{c,d}	15,594 ^{y,x}

Tests of significance were made between groups with the same letter:-

a	0.05 < P < 0.10	v	P < 0.001
b	0.40 < P < 0.50	y	P < 0.001
c	0.20 < P < 0.30	w	0.30 < P < 0.40
d	0.10 < P < 0.20	x	0.80 < P < 0.90

PART 36.3 The effect of sodium taurochenodeoxycholate on calcium transportMETHOD

Sodium taurochenodeoxycholate (gift from Professor E.S. Holdsworth) was added to $^{45}\text{Ca}^{2+}/^{40}\text{Ca}^{2+}$ solution (4 mg Ca^{2+} /ml, 10 μCi /ml) as a final concentration of 7mM. 500 μl $^{45}\text{Ca}^{2+}/^{40}\text{Ca}^{2+}$ solution with or without the bile salt was placed in the duodenal loop of rachitic chicks for 20 min as described in 1.4.1 and 1.4.2. The mucosal cells were treated as described in 1.5.1, 1.5.2 and 1.5.3.1. Bone and blood samples were taken at 20 min and treated as described in 1.7.3.2. and 1.7.4.1.

RESULTS

From Table 52A it can be seen that the bile salt has not caused an increase in cellular Ca^{2+} content but has enhanced the transport of Ca^{2+} across the cell, as shown by a 3 fold rise in plasma $^{45}\text{Ca}^{2+}$ and 2 fold increase in bone $^{45}\text{Ca}^{2+}$ after 20 min loading. The cellular $^{45}\text{Ca}^{2+}$ distribution does not appear to have been altered by the presence of bile salt in the lumen, nor has the cellular $^{40}\text{Ca}^{2+}$ concentration increased from that for the rachitic chick (Table 52B).

TABLE 52

The effect of sodium taurochenodeoxycholate on calcium transport by the duodenum

500 μ l $^{45}\text{Ca}^{2+}/^{40}\text{Ca}^{2+}$ solution (2 mg, 5 μ Ci) with or without 7mM-Na taurochenodeoxycholate was placed in duodenal loop of starved rachitic chick for 20 min (1.4.1 and 1.4.2). The mucosal cells were isolated and homogenized in Krebs-Ringer Phosphate + 0.25M-sucrose (1.5.1 and 1.5.2). The homogenate was fractionated as described in 1.5.3.1. Bone and blood samples were taken at 20 min and treated as described in 1.7.3.2 and 1.7.4.1. The results are the average of 3 experiments with 2 chicks in each group expressed as per duodenum.

A

	MUCOSAL CELLS	PLASMA	TIBIA
Na taurochenodeoxycholate	C.P.M./ duodenum	C.P.M./ ml plasma	C.P.M./ ashed tibia
-	1,726,343	586	22,918
+	1,479,966	1,502	50,532

B

Fraction	Na taurochenodeoxycholate	Protein mg	$^{40}\text{Ca}^{2+}$ μ g	C.P.M.	C.P.M./ $^{40}\text{Ca}^{2+}$ μ g
Main	-	54	263	924,297	3,513
Residue	+	53	219	737,862	3,375
Crude	-	52	212	792,955	3,742
Mitochondria	+	53	192	726,720	3,785
Microsomes	-	20	8	9,011	1,119
	+	23	9	14,617	1,740
Final	-	54	8	81	10
Supernatant	+	63	9	767	89

PART 46.4 The effect of the ethacrynic acid on calcium transport in the live chickMETHOD

Starved rachitic and vitamin D₃-replete chicks (800 I.U. vitamin D₃, 16 h before experiment) were dosed with 1 ml propylene glycol with or without 120 mg EDECRIL (50 mg ethacrynic acid) orally 4 h before the experiment, or 200 μ l ethanol with and without 40 mg ethacrynic acid was injected intravenously (wing vein) 1 h before the experiment. The duodenal loop was loaded for 20 min as described in 1.4.1 and 1.4.2. The mucosal cells were isolated as described in 1.5.1. These cells were weighed and placed in 5 ml 0.5M-HClO₄ for 24 h, mixed several times during this period and then centrifuged. 100 μ l of supernatant were counted. A bone sample was taken from each chick and treated as described in 1.7.4.2.

RESULTS

From Table 53, one can see that both oral and intravenous administration of ethacrynic acid has reduced Ca²⁺ transport by about 50%. However the effect of vitamin D₃ on Ca²⁺ transport has not been eliminated. The content of ⁴⁵Ca²⁺ in rachitic and vitamin D treated chicks was increased by 50%, while the ⁴⁵Ca²⁺ content of bones was reduced by a similar amount. The effects obtained with ethacrynic acid were statistically highly significant.

TABLE 53

The effect of ethacrynic acid on calcium transport by the duodenum

Starved rachitic and vitamin D₃-replete chicks were dosed with 1 ml propylene glycol with or without 50 mg ethacrynic acid (120 mg Edecril) orally 4 h before the experiment, or 200 μ l ethanol with or without 40 mg ethacrynic acid 1 h before the experiment. The duodenal loops were loaded with 500 μ l $^{45}\text{Ca}^{2+}/^{40}\text{Ca}^{2+}$ solution (2mg, 5 μ Ci) for 20 min (1.4.1 and 1.4.2). The mucosal cells were isolated (1.5.1), weighed and added to 5 ml 0.5M-HClO₄ for 24 h, mixed several times during this period, and then centrifuged and counted 100 μ l of the supernatant (1.8.4). A bone sample was taken from chicks and treated as described in 1.7.4.2. The results are the average for 4 chicks.

Tests of significance were made between groups with the same letter:-

a	0.30	<	P	<	0.40	i	0.40	<	P	<	0.50
b	0.70	<	P	<	0.80	j	0.30	<	P	<	0.40
c			P	<	0.001	k	0.02	<	P	<	0.05
d	0.05	<	P	<	0.10	l	0.001	<	P	<	0.01
e	0.001	<	P	<	0.01	m	0.001	<	P	<	0.01
f	0.001	<	P	<	0.01	n	0.001	<	P	<	0.01
g	0.01	<	P	<	0.02	o	0.02	<	P	<	0.05
h	0.10	<	P	<	0.20	r	0.001	<	P	<	0.01

Table 53

Type of chick	oral dose 50 mg			
	C.P.M.			
	/g wet weight of cells		/whole tibia	
	-*	++	-	+
Vitamin D ₃ - treated	377,992 ^{a,c}	816,183 ^{b,c}	16,175 ^{e,g}	5,546 ^{f,g}
Rachitic	467,160 ^{a,d}	860,525 ^{b,d}	2,385 ^{e,h}	1,226 ^{f,h}

Type of chick	intravenous dose 40 mg			
	C.P.M.			
	/g wet weight of cells		/whole tibia	
	-*	++	-	+
Vitamin D ₃ - treated	941,156 ^{i,k}	2,005,240 ^{j,k}	36,065 ^{m,o}	18,065 ^{n,o}
Rachitic	1,127,287 ^{i,l}	2,475,198 ^{j,l}	6,155 ^{m,r}	3,077 ^{n,r}

PART 56.5 The effect of temperature on calcium transport in the everted ileal sacMETHOD

The ilea (the distal third of the small intestine) of starved rachitic and vitamin D₃-replete chicks were everted and incubated as described by Sallis and Holdsworth (1962a). The everted sacs were incubated for 15 min at 0°C or 30°C in 5 ml Krebs-Hessleit-bicarbonate buffer (Dawson and Elliott, 1959) containing 20mM-glucose and 1mM- $^{45}\text{CaCl}_2$ / $^{40}\text{CaCl}_2$ (25 μCi). The sac (serosal surface) contained 1 ml of same solution but with $^{40}\text{Ca}^{2+}$. After 15 min, the mucosal surface of the sac was washed by immersion in the buffer containing $^{40}\text{Ca}^{2+}$, and the sac was reincubated with fresh serosal and mucosal fluids which were at the same concentration as above, but made with $^{40}\text{Ca}^{2+}$. After the second incubation at 30°C for 15 min, the $^{45}\text{Ca}^{2+}$ released to the serosal and mucosal fluid and that remaining in the tissue after digestion of the sac was determined (1.7.5).

RESULTS

The amount of $^{45}\text{Ca}^{2+}$ taken up by the everted sacs at 0°C was approximately half that taken up at 30°C (Table 54). However of the $^{45}\text{Ca}^{2+}$ taken up at 0°C, only a very small amount was translocated to the serosal fluid, but if the temperature was raised to 30°C then more $^{45}\text{Ca}^{2+}$ was transported to the serosal fluid. Everted sacs that were incubated at 30°C for both the first and second periods show a rate of translocation 3 times greater for vitamin D-replete sacs

as compared to rachitic sacs. The rate of release of $^{45}\text{Ca}^{2+}$ to the serosal fluid was the same during the second 15 min period as in the first 15 min period at 30°C . The amount of $^{45}\text{Ca}^{2+}$ left in the tissue at 30 min was the same for rachitic and vitamin D_3 -replete sacs when the uptake of $^{45}\text{Ca}^{2+}$ occurred at 0°C . Vitamin D_3 -replete sacs incubated at 30°C throughout the experiment, accumulated more $^{45}\text{Ca}^{2+}$ in the tissues than rachitic sacs.

TABLE 54

Uptake and transport of calcium by everted ileum at 0°C and 30°C

The ilea from starved rachitic and vitamin D-replete chicks were everted and incubated as described by Sallis and Holdsworth (1962a). The everted sacs were incubated for 15 min at 0°C or 30°C in 5 ml Krebs-Hensleit bicarbonate buffer (Dawson and Elliott, 1959) containing 20mM-glucose and 1mM $^{45}\text{CaCl}_2$ (5 $\mu\text{Ci/ml}$). The sac contained 1 ml of the same solution but with $^{40}\text{Ca}^{2+}$. After 15 min the mucosal surface of the sac was washed by immersion in buffer containing $^{40}\text{Ca}^{2+}$, and the sac was reincubated with fresh serosal and mucosal fluid but made with $^{40}\text{Ca}^{2+}$. After 30 min incubation for 15 min the $^{45}\text{Ca}^{2+}$ released from mucosal and serosal fluid and $^{45}\text{Ca}^{2+}$ remaining in the sacs was determined (Sallis and Holdsworth, 1962a).

Type of Chick	First incubation C.P.M.		Second incubation C.P.M.		C.P.M. in ileum at 30 min
	in serosal fluid	lost by mucosal fluid	in serosal fluid	gained by mucosal fluid	
Conditions	15 min at 0°C		15 min at 30°C		
Vitamin D ₃ -treated	4,920*	2,329,247	106,480*	330,300	401,600
Rachitic	2,170*	2,181,666	98,455*	410,950	409,500
Conditions	15 min at 30°C		15 min at 30°C		
Vitamin D ₃ -treated	284,310+	4,803,791	297,360+	412,825	1,096,000+
Rachitic	94,470+	4,441,988	116,730+	322,275	618,300+

The results are average for six sacs.

* R/D difference significant $P = 0.2$

+ R/D difference significant $P = 0.01$

Other R/D differences not significant.

PART 66.6 The efflux of calcium from the plasma to lumenMETHOD

200 μ l, 20 μ Ci carrier free $^{45}\text{Ca}^{2+}$ solution was injected intraperitoneally into rachitic and vitamin D_3 -replete chicks. Ten and twenty min after the injection, blood samples were taken and treated as described in 1.7.3.1. At 20 min, the small intestine proximal end of the duodenum to distal end of the ileum was washed out with 20 ml 0.154M-NaCl in situ. The washings were collected, made up to 40 ml with distilled water, acidified to 0.5M- HClO_4 and centrifuged. The supernatant was decanted off and 1 ml counted in 20 ml Triton X100 as described 1.8.4. The small intestine was removed, washed externally and then digested as described in 1.7.5. The digest residue was dissolved in 10 ml 0.5M- HClO_4 and estimated for $^{45}\text{Ca}^{2+}$ as described 1.8.4. A bone sample was taken at the end of 20 min period and treated as described in 1.7.4.1.

RESULTS

There appears to be no greater diffusion of $^{45}\text{Ca}^{2+}$ from plasma to lumen in vitamin D-replete chicks as compared to rachitic chicks as shown by the $^{45}\text{Ca}^{2+}$ found in the washings of the intestine (Table 55). The blood, gut digests and bones from vitamin D and rachitic chicks do not show any significant difference in their $^{45}\text{Ca}^{2+}$ content.

TABLE 55

The efflux of calcium from plasma to intestinal lumen

200 μ l $^{45}\text{Ca}^{2+}$ (20 μCi , carrier free) was injected intraperitoneally into starved, vitamin D-replete and rachitic chicks. After 20 min, the proximal to distal end of the small intestine (duodenum through to the ileum) was washed out with 20 ml 0.154M-NaCl. The washings were collected, made up to 40 ml with distilled water, acidified to 0.5M-HClO₄ and centrifuged. 1 ml of supernatant was counted in 20 ml Triton-X100. The small intestine was removed, washed externally and then digested as described in 1.7.5. The digest residue was dissolved in 10 ml 0.5M-HClO₄ and 100 μ l was counted in Bray's Phosphor (1.8.4). Blood samples were taken at 10 min and 20 min and treated as described in 1.7.3.1. Bone samples were taken at 20 min and treated as described in 1.7.4.1. The results are the average for 9 chicks expressed as per chick.

Type of chick	C.P.M./ ml plasma at 10 min	C.P.M./ ml plasma at 20 min	C.P.M./ ashed tibia at 20 min	C.P.M./ total washings	C.P.M./ gut digest
Vitamin D ₃ - treated	13,039	10,755	233,576	39,236	327,676
Rachitic	16,920	10,766	286,047	59,236	394,112

None of the results are significantly different between vitamin D₃-treated chick and rachitic chick samples.

Discussion:

In this chapter an attempt was made to find evidence of an effect of vitamin D on the membrane enclosing the cell.

Wasserman and coworkers have shown that when vitamin D₃ was given to chicks, the onset of enhanced Ca²⁺ transport was associated with the formation of CaBP, which may be located at the lumen surface (Ebel et al 1969; Taylor and Wasserman, 1970). When seven times the quantity of CaBP present in normal chick intestine was placed in the duodenum together with ⁴⁵Ca²⁺, no increased translocation of Ca²⁺ was observed. Thus isolated CaBP on the outside of the cell was not effective in Ca²⁺ transport, although it is possible that had the CaBP been part of the membrane structure it might have been more effective in this process.

It has been reported that the Ca²⁺ binding sites on mitochondria are inhibited by ruthenium red (Vasington et al, 1972). However ruthenium red had no effect on Ca²⁺ transport from the duodenum of either rachitic or vitamin D₃-treated chicks, although the microvillus region of the mucosal cells was deeply stained. This suggests that the sites of Ca²⁺ transport are very different in mitochondria and microvilli.

Webling and Holdsworth (1965; 1966) found that bile salts would increase Ca²⁺ translocation from intestinal lumen to plasma and bone. Sodium taurochenodeoxycholate was found to increase ⁴⁵Ca²⁺ transport in rachitic chicks approximately two fold (Table 52A), but did not cause any alteration in the amount or distribution of ⁴⁵Ca²⁺ within the mucosal cell. This must mean that the mechanism

for removing Ca^{2+} from the cell at the serosal surface maintains the Ca^{2+} content of the cell constant, although more Ca^{2+} is passing through the cell due to the increased permeability of the lumen surface brought about by bile salt or when vitamin D_3 was given.

Ethacrynic acid has been shown to inhibit a $\text{Ca}^{2+}/\text{Na}^{+}$ ATPase located on the basement membrane of the serosal surface (Birge et al, 1972). When given to chicks that were transporting $^{45}\text{Ca}^{2+}$ in vivo, ethacrynic acid did not appear to inhibit the entry of Ca^{2+} into the cell. The inhibitor did however interfere with the transport of Ca^{2+} out of the cell, causing accumulation of Ca^{2+} within the cell, and less Ca^{2+} appeared in the bones. This result, taken with the observation of Birge et al (1972), suggests that Ca^{2+} is actively pumped out of the mucosal cell into the extracellular fluid and that ethacrynic acid inhibits this $\text{Ca}^{2+}/\text{Na}^{+}$ operated pump.

Further evidence for a metabolically operated pump at the serosal surface was obtained by the experiment with isolated everted sacs of chick ileum. At 0°C , Ca^{2+} was able to enter the mucosal cell but was unable to be transported to the serosal fluid. On raising the temperature of these sacs to 30°C the accumulated $^{45}\text{Ca}^{2+}$ was pumped out in amounts comparable to everted sacs that had been kept at 30°C throughout the transport experiment. Thus Ca^{2+} can enter the mucosal cell without metabolic energy being required, but a metabolically operated pump is required to remove Ca^{2+} via the serosal surface.

Experiments done in vitro invariably show a greater accumulation of Ca^{2+} within mucosal cells of vitamin D_3 -treated as

compared to rachitic cells. This difference was found when the Ca^{2+} content of mucosal cells from in vivo experiments was determined. It may be that when a loop of intestine is isolated, the mucosal cells are deprived of their oxygen supply and the metabolically operated serosal Ca^{2+} pump is then operating less efficiently, causing Ca^{2+} to accumulate especially in the vitamin D_3 -treated cells.

Vitamin D_3 has been reported by a number of experimenters to increase the permeability of the mucosal surface of the cells of the duodenum (Harrison and Harrison, 1965; Wasserman et al, 1966). However this was not found when $^{45}\text{Ca}^{2+}$ was injected intraperitoneally, as the luminal contents of the vitamin D_3 -treated chicks and rachitic chicks contained the similar amounts of $^{45}\text{Ca}^{2+}$. Thus vitamin D has neither increased the permeability of mucosal cells from plasma to mucosal cell, nor from cell to lumen.

CHAPTER 7FINAL CONCLUSION AND A MODEL FOR CALCIUM TRANSPORT

In this final chapter, an attempt will be made to integrate the findings of the work done in earlier chapters of this thesis, then these new observations will be used to extend the previous published models of the mechanism of Ca^{2+} translocation in the intestine.

When Ca^{2+} is being absorbed from the lumen of the small intestine it has to pass across the epithelial cells. It will be assumed that the amount of Ca^{2+} passing the tight junction that joins the epithelial cells at their apical borders, is insignificant. Thus the Ca^{2+} being absorbed has (1) to traverse the border at the luminal surface which is made up of microvilli, (2) to cross the cell with its complement of organelles and (3) to leave the cell via the lateral and basement membranes into the extracellular space. Therefore there are many places at which 1, 25 DHCC can exert its effect. In this discussion an effect attributed to vitamin D_3 will be understood to be due to the true hormone; 1, 25 DHCC.

It has been well established that vitamin D enhances the overall translocation of Ca^{2+} across the intestine (reviewed Rasmussen and DeLuca, 1963; DeLuca, 1967; Norman, 1968; Wasserman, 1968). In chapter 2 it was shown that although vitamin D increased the flow of Ca^{2+} three fold to five fold across the chick duodenum, the Ca^{2+} content of the mucosal cells for rachitic and vitamin D_3 -treated cells was the same 20 min after placing 2 mg of Ca^{2+} in the duodenum. Under these conditions the concentration of Ca^{2+} left in

the lumen of rachitic or vitamin D₃-treated chicks would not be greatly different. Since in the vitamin D₃-treated duodenum three times as much Ca²⁺ is entering the mucosal cells one would expect a higher content in these cells than the rachitic cells. However since the two types of cell were found to contain similar amounts of Ca²⁺, it seems as if the vitamin D₃-treated cells are able to remove this Ca²⁺ more rapidly than the rachitic cells. This was shown by a 'turnover' experiment in which rachitic and vitamin D₃-treated chick duodena were loaded to the same extent with ⁴⁵Ca²⁺, then the disappearance of ⁴⁵Ca²⁺ from the duodenal cells studied in vivo (Table 10). The vitamin D₃-treated cells lost more ⁴⁵Ca²⁺ than did the rachitic cells. This experiment shows that apart from any effect of vitamin D₃ on the entry of Ca²⁺ into the mucosal cells, the vitamin also causes a more rapid turnover of Ca²⁺ recently absorbed by the cell. The slow turnover of Ca²⁺ by rachitic mucosal cell may account for the fact that when large amounts of Ca²⁺ are being absorbed in vivo over a period of 60 min or longer, then rachitic intestine cells accumulate more Ca²⁺ than do vitamin D₃-treated cells (Table 7 and Neville and Holdsworth, 1968).

An attempt was made to discover the state of Ca²⁺ during its passage through the epithelial cells of the intestine. Careful fractionation studies by conventional methods, using homogenation of the mucosal cells in 0.25M-sucrose at 0°C, gave results suggesting that the concentration of 'free' Ca²⁺ in the cell is very small, while most of the Ca²⁺ was present in the mitochondria with lesser amounts in the nuclei and microsomes (Hamilton and Holdsworth, 1969). These

results agree with many observations made usually with rat liver or kidney mitochondria (Thiers and Vallee, 1957; Vasington and Murphy, 1961 and 1962; Drahota et al, 1965; Wester, 1965; Patriarca and Carafoli, 1968).

However it was shown in chapter 3 that an homogenate of duodenal mucosal cells prepared in 0.25M-sucrose at 0°C would take up an amount of Ca^{2+} equivalent to 400 μg per duodenum, and this Ca^{2+} was found in the organelles particularly in the mitochondria. Therefore even if 'free' Ca^{2+} exists within intestinal cells in vivo, this method of preparation would not be suitable to detect it. A number of inhibitors were investigated to find conditions that would prevent the redistribution of Ca^{2+} during homogenation of the mucosal cells. The inhibitors were studied for their effect on uptake or release of Ca^{2+} from isolated mitochondria or cell homogenate, and for their effect on the pattern of distribution of Ca^{2+} amongst the organelles when duodena were loaded with Ca^{2+} in vivo and homogenized at 0°C. Most inhibitors used were unsatisfactory, since those that inhibited uptake of Ca^{2+} by mitochondria at 0°C, also caused the release of Ca^{2+} accumulated by this organelle. One substance proved useful and that was ruthenium red, which prevented Ca^{2+} uptake by mitochondria or a cell homogenate, and did not release endogenous Ca^{2+} . Unfortunately ruthenium red combined with organelles and with some cell proteins, causing them to centrifuge down at low speeds (e.g. 800g for 10 min). This made it impossible to study the pattern of distribution of Ca^{2+} within the cell, but did make it possible to assess the amount of Ca^{2+} present in the soluble phase of the cell. Cells

loaded with Ca^{2+} in vivo, then cooled and homogenized in 0.25M-sucrose containing excess ruthenium red, contained 14% of their total Ca^{2+} in the cytoplasmic fraction. This would amount to $3 \times 10^{-4} \text{M-Ca}^{2+}$ in the soluble phase of the duodenal cell (calc. from Table 31A). The water content of mucosal cells from duodena of chicks similar to those used in this work was determined by correcting the loss of weight on drying at 110°C by subtracting the extracellular water determined by [^{14}C] polyethylene glycol (personal communication by Prof. E.S. Holdsworth). The wet weight of cells averaged 1.63g, the dry weight 0.27g, the protein content 200 mg and the cell water was calculated to be 1.25ml.

Since during homogenation at 0°C , Ca^{2+} can be taken up by mitochondria, it seemed possible that mere cooling of the cells to 0°C might cause an altered pattern of Ca^{2+} distribution. A homogenate of $^{45}\text{Ca}^{2+}$ loaded mucosal cells was prepared at 0°C and brought to 22°C ; 31% of the $^{45}\text{Ca}^{2+}$ was released into the soluble phase. This experiment is similar to work reported by Rasmussen et al (1963) on isolated intestinal villi and by Holdsworth (1965) on isolated mucosal cells. The cell homogenate prepared in 0.25M-sucrose at 22°C and centrifuged at 78,000g for 30 min contained 18% of the total Ca^{2+} in the supernatant (Table 33). Ruthenium red was shown to prevent Ca^{2+} uptake by isolated mitochondria or a cell homogenate at 22°C and did not cause release of Ca^{2+} from mitochondria previously loaded with $^{45}\text{Ca}^{2+}$ in vivo (ref. chapter 3). When mucosal cells loaded with $^{45}\text{Ca}^{2+}$ in vivo were scraped off at room temperature and homogenized in 0.25M-sucrose containing excess ruthenium red, the 78,000g supernatant contained

32% of the total Ca^{2+} . Similar amounts of soluble (cytoplasmic) Ca^{2+} was found in supernatant from rachitic or vitamin D_3 -treated duodenal cells. If these estimates of the soluble Ca^{2+} are correct the concentration of soluble Ca^{2+} in the duodenum mucosal cells during absorption is approximately $2 \times 10^{-3} \text{M-Ca}^{2+}$. Such a concentration of soluble Ca^{2+} is contrary to the generally accepted values which have always been obtained by cooling the tissue to 0°C and homogenizing at approximately 2°C . Homogenation of tissue at 0°C in the presence of ruthenium red gave rise to values of $3 \times 10^{-4} \text{M-Ca}^{2+}$. Ruthenium red prevents the uptake of Ca^{2+} by mitochondria at 0°C and at 30°C and does not release Ca^{2+} from mitochondria at these temperatures. Homogenation of mucosal cells in plain 0.25M -sucrose at 22°C instead of 0°C , led to 18% of the total Ca^{2+} being found in the soluble fraction, compared with 2% at 0°C . Whether the Ca^{2+} present in the cytoplasm at 22°C or above is free ionic Ca is not known. It is possible that Ca^{2+} weakly bound to some ionic component can be displaced by ruthenium red. Alternatively Ca^{2+} might be present in or on microtubules (Cardell, Badenhauser and Porter, 1967), which, on homogenation in the presence or absence of ruthenium red, release their Ca^{2+} into the soluble phase. The Ca^{2+} released in the absence of ruthenium red could then be taken up by the mitochondria, thus lowering the amount of Ca^{2+} in the soluble phase. Investigations of cell homogenates prepared in 0.25M -sucrose at 0°C and 28°C by centrifugation in a zonal centrifuge lends support to the concept that at 28°C , and perhaps in the living cell, mitochondria are the organelles that contain a large proportion of the Ca^{2+} but at these temperatures a considerable

amount of Ca^{2+} remains in the soluble phase. Whereas at 0°C the soluble Ca^{2+} has been taken up by the mitochondria.

The picture that emerges from these results is that in cells in vivo, approximately two-thirds of the cell Ca^{2+} is probably in the organelles, mainly the mitochondria, whilst one third of the Ca^{2+} is in the cytoplasm. Equilibrium between the Ca^{2+} in the organelles and soluble Ca^{2+} is probably maintained by the metabolic activity of the cell. It was shown that mucosal cells from vitamin D_3 -treated chicks 'turnover' their Ca^{2+} at a greater rate than rachitic chicks. In searching for a reason for this effect of vitamin D, it was found that the CaBP causes the release of Ca^{2+} from either rachitic or vitamin D_3 -replete mitochondria. This release took place at 30°C , and more Ca^{2+} was released than could be accounted for by the binding activity of the CaBP. The amount of CaBP present in one chick duodenum, as used in this study, was approximately 7 nmoles (assuming a molecular weight of 28,000). When this amount of CaBP was added to mitochondria in the system used in this work, 28 nmoles of Ca^{2+} were released in 15 min at 30°C . Under the conditions of these experiments the 7 nmoles of CaBP bound approximately 3 nmoles Ca^{2+} (chelex test at 10^{-5}M Ca^{2+}). In other words this release was not due solely to the sequestering of the Ca^{2+} such as would happen with 10^{-3}M -EGTA. Thus the slow turnover of Ca^{2+} in rachitic mucosal cells may be due to the absence of CaBP in these cells.

For reasons discussed in chapter 5 it is considered unlikely that PTH or various adenine nucleotides or prostaglandin have any physiological role in the release of Ca^{2+} from mitochondria.

If CaBP plays an intracellular role it would be expected that it exists in the cytoplasm. When vitamin D₃-treated mucosal cells were homogenized in 0.25M-sucrose at 0°C and a 78,000g supernatant prepared, most of the vitamin D₃-induced Ca-binding activity was found in this supernatant (chapter 4). Taylor and Wasserman (1970a) examined the mucosal cells by immunofluorescent methods for the location of the CaBP and claimed that fluorescence was seen in the brush border, the goblet cells and lamina propia area. This technique suffers from the defect that a soluble protein would tend to migrate to cell surfaces when the frozen section is thawed before the application of the fluorescent antiserum, and its intracellular location might be missed. Certainly homogenation in 0.25M-sucrose used in this thesis would release CaBP from goblet cells and possibly from the brush border or other organelles if only lightly bound. Since it seems likely that the CaBP is made within the mucosal cell it might be available to effect the intracellular metabolism of Ca²⁺. Taylor and Wasserman (1970a) noted that on a few occasions a very diffuse distribution of the fluorescent antibody to CaBP throughout sections of duodenal tissue occurred. This may show that the CaBP may be distributed throughout the mucosal cell.

Two suggestions have been put forward to explain an effect of CaBP on Ca²⁺ transport. Wasserman et al (1969) have suggested that the CaBP present in or at the brush border region could facilitate diffusion of Ca²⁺ into the cell. A similar suggestion by DeLuca (1971) postulates that CaBP secreted from the goblet cells into the lumen facilitates the entry of Ca²⁺ into the interior of the cell. To test

this last theory a seven fold excess of pure CaBP was placed together with $^{45}\text{Ca}^{2+}$ into rachitic duodena for 1 h. The rate of transport of $^{45}\text{Ca}^{2+}$ into the mucosal cells and into bone were not significantly different than using rachitic duodena alone (chapter 6). Duodena were carefully washed out, then 500 μl 0.154M-NaCl left in a ligated loop in vivo for 20 min and this saline was tested for the presence of CaBP by the chelex binding test. No Ca^{2+} binding activity was detected in chicks given 800 I.U. vitamin D_3 for 68 h. In this experiment the vitamin D_3 -treated mucosal cells contained their normal complement of CaBP. Thus if CaBP facilitates Ca^{2+} transport at the brush border, it must be an integral part of the border or act on the inside of the cell membrane. When ruthenium red was placed in the duodenal loop it heavily stained the outer surface of the cell (chapter 6) but did not penetrate the cell. Ruthenium red in the lumen did not alter the in vivo translocation of Ca^{2+} in either rachitic or vitamin D_3 -treated chicks. Thus the uptake of Ca^{2+} by the microvilli surface is very different from the mitochondrial surface where ruthenium red competes strongly for the Ca^{2+} binding sites.

The mucosal border of rachitic chicks can be made more permeable to Ca^{2+} , as shown by Webbing and Holdsworth (1965), by having certain bile salts or sodium lauryl sulphate present in the lumen. When sodium taurochenodeoxycholate was used in vivo, it increased the overall translocation of Ca^{2+} two fold, but had no effect on the cellular Ca^{2+} content or on the distribution of Ca^{2+} within the cell. It appears that the intracellular Ca^{2+} content is maintained at a constant

level by the operation of some mechanism at the serosal surface of the cell.

The presence of a metabolically operated pump at the serosal surface has been inferred from consideration of the concentration in plasma, 3mM in chicks, to that in the cell which is believed to be less than this value and also from experiments of Schachter et al (1960; 1961; 1966) where Ca^{2+} was moved against a concentration gradient in everted sacs of rat intestine. Support for the idea of a serosal pump came with the work of Birge et al (1972), who showed that ethacrynic acid, which inhibited Ca^{2+} transport by everted sacs of intestine, would also inhibit a $\text{Ca}^{2+} / \text{Na}^{+}$ ATPase located at the lateral and basement membranes. (The ATPases of the brush border were not inhibited by ethacrynic acid.) In chapter 6 it was shown that ethacrynic acid partially inhibited Ca^{2+} transport in both rachitic and vitamin D_3 -treated chicks in vivo. Ethacrynic acid caused an increase in the Ca^{2+} content of the mucosal cells, showing that it inhibited efflux from the cell but not the influx.

When everted sacs of chicks ileum were placed in ice-cold Krebs-Henseleit bicarbonate medium containing $10^{-3}\text{M}-\text{Ca}^{2+}$, the cell took up a considerable amount of Ca^{2+} at 0°C , but at this temperature did not transport the Ca^{2+} into the serosal fluid. On raising the temperature of the system to 30°C the Ca^{2+} accumulated at 0°C was transported to the serosal fluid (chapter 6). This shows that a temperature sensitive pump is located at the serosal surface which pumps Ca^{2+} out of the cell. This pump probably requires Na^{+} (Martin and DeLuca, 1969; Birge et al, 1972).

The plasma to lumen flux of Ca^{2+} is unaffected by treatment of the chick with vitamin D as shown by experiment in chapter 6. In experiments with everted gut sacs, Schachter (1963), Holdsworth (1965) and Sallis and Holdsworth (1962) proposed that vitamin D had no effect on the transfer of Ca^{2+} from serosa to mucosa. However from in vivo experiments done by Wasserman and coworkers (Wasserman, 1963; Wasserman and Kallfelz, 1962; Wasserman et al, 1966), they inferred that the transfer of Ca^{2+} from the mucosal cell to the lumen was accelerated by the vitamin. However in a similar experiment to those of Wasserman's, neither the plasma to cell nor cell to lumen transfer of Ca^{2+} was enhanced by vitamin D. Plasma to lumen flux would be difficult to measure as the pump at the serosal surface of the cell would return most of the Ca^{2+} entering at this surface to the plasma before it could reach the luminal side of the cell. Also the $^{45}\text{Ca}^{2+}$ that did enter the lumen of the vitamin D_3 -replete chick would be returned faster to plasma than that of the rachitic chick, as studied over a period of 20 min.

One aspect of the effect of vitamin D_3 which has not been thoroughly investigated in this thesis, is the effect of the vitamin on the phosphatases of the mucosal cell. It was observed early in this work (1968 unpublished) that vitamin D_3 -treated mucosal cells always had a higher content of alkaline phosphatase than rachitic cells (ref. chapter 2). Martin and DeLuca (1969) reported that vitamin D_3 induced the formation of a Ca^{2+} stimulated ATPase. However Haussler et al (1970), Nagode et al (1970), Holdsworth (1970) and Norman et al (1970) all came to the conclusion that the enzyme

activity that increased after giving vitamin D₃ could be described as alkaline phosphatase, or inorganic pyrophosphatase, or an ATPase according to the conditions of the assay. Inhibitors that inhibited one activity, decreased all three activities. Phenylalanine that inhibits alkaline phosphatase by about 80%, at $2 \times 10^{-2} M$, had no inhibitory effect on Ca^{2+} transport at $5 \times 10^{-2} M$ when using everted chick intestine (Holdsworth, 1970). Thus the role of the enzyme with these properties on Ca^{2+} metabolism remains an unsolved but interesting observation, since no other enzyme activities have been found to vary with the ability to absorb Ca^{2+} .

Model for calcium transport

Many models have been proposed but no conclusive system has been established for the enhancement of Ca^{2+} transport by vitamin D. Much of the earlier evidence is contradictory. One model proposed the existence of active pumps at the serosal and mucosal surfaces of the mucosal cell (Schachter et al, 1966), a second incorporated two active pumps as well as diffusion mechanisms (Holdsworth, 1965), a third had an active pump at the serosal border of the cell, supplemented by passive diffusion at the mucosal surface (Wasserman, 1968), and yet another had an active mucosal pump and exchange diffusion process at the serosal surface (DeLuca, 1971).

To fit the data presented in this thesis, a model for Ca^{2+} transport enhanced by vitamin D is shown in figure 14.

In this model it is proposed that Ca^{2+} passage through the mucosal surface of the cell is by facilitated diffusion. This process was shown not to be dependent on oxidative metabolism (Harrison and

Harrison, 1960, 1965; Wasserman, 1963a; Schachter et al, 1966), independent of temperature (chapter 6), not inhibited by ethacrynic acid or ruthenium red (chapter 6), not readily saturable with Ca^{2+} (chapter 2), but enhanced by a bile salt, sodium taurochenodeoxycholate (chapter 6) and by prior treatment of the animal with vitamin D_3 (chapters 2, 3; 6; Harrison and Harrison, 1960; 1965; Wasserman, 1963; Schachter et al, 1965). Vitamin D induces the synthesis of CaBP and also increases the activity of a phosphatase in the mucosal cell. Whether these two components of the cell are linked in some way to facilitate diffusion is not known, but CaBP, possibly by acting as a membrane carrier, could aid diffusion. Complexing with the binding protein situated on the inside of the brush border might reduce the activity (activity coefficient) of Ca^{2+} within the microvilli, thus favouring an inward flow of Ca^{2+} down a gradient.

The Ca^{2+} content of the cell is not increased by vitamin D_3 in vivo over a range of 5mM to 100mM- Ca^{2+} studied or by the bile salt. The transitory Ca^{2+} is not totally exchangeable with the residual Ca^{2+} of the mitochondria as was shown in the turnover experiment, where it was the recently absorbed $^{45}\text{Ca}^{2+}$ that was released. Once Ca^{2+} enters the cell, mitochondria start to sequester this transitory Ca^{2+} so to maintain the cytoplasmic Ca^{2+} between 1-2mM- Ca^{2+} during the loading state. Thus the mitochondria function as storage organs and come to equilibrium with the cytoplasmic Ca^{2+} depending on the concentration of Ca^{2+} in the lumen and the length of time that Ca^{2+} is present in the lumen and the operation of the serosal Ca^{2+} pump. Mitochondria may also be involved in the translocation of Ca^{2+} within the cell, as

suggested by Sampson et al (1970). Ernster and Lindberg (1968) and Frederic (1958) have observed mitochondria to move about within the cell.

The observation that CaBP can cause Ca^{2+} to be released from mitochondria (Hamilton and Holdsworth, 1970 ; chapter 5) suggests that this vitamin D_3 -induced protein controls the equilibrium between the cytoplasm and the mitochondria. Certainly in the absence of the CaBP (i.e. rachitic chicks), turnover of Ca^{2+} by the mitochondria was less and after 60 min of loading, a larger amount of Ca^{2+} was present in mitochondria of rachitic chicks than in the vitamin D_3 -replete organelles. This suggests that the CaBP, by increasing Ca^{2+} turnover, makes more Ca^{2+} available for the serosal Ca^{2+} pump.

A pump to remove Ca^{2+} from the mucosal cell is probably located at the lateral and basement membranes of the cell. This would be necessary on the accepted values of intracellular Ca^{2+} $10\ \mu\text{M}$ to $1\ \mu\text{M}$ - Ca^{2+} (Thiers and Vallee, 1957; Drahota et al, 1965) to pump the Ca^{2+} uphill to the extracellular fluid which is 2 to 3mM- Ca^{2+} . If this figure of 1 to 2mM- Ca^{2+} suggested in this thesis is accepted, then the pump would still be required to transport Ca^{2+} from the cell to plasma. Evidence for the existence of the pump is (1) that it is sensitive to temperature and virtually ceases at 0°C (chapter 6); (2) that it is inhibited by ethacrynic acid (chapter 6), (3) that a $\text{Ca}^{2+}/\text{Na}^+$ ATPase has been located in the lateral and basement membranes of the cell that is inhibited by ethacrynic acid (chapter 6 ; Birge et al, 1972) and (4) that it required Na^+ (Martin and DeLuca, 1969).

When, under the stimulus of vitamin D, three or more times as much Ca^{2+} is passing through the mucosal cell than through a rachitic cell, the serosal pump is able to keep the intracellular Ca^{2+} concentration the same as in the rachitic cell up to 20 min. This means that the pump, (1) has a capacity in excess of the normal amount of Ca^{2+} passing through the cell and maintains Ca^{2+} concentration at a constant level or (2) the pump can be activated by vitamin D in such a way that the total amount of Ca^{2+} passing across the microvilli can be pumped out of the cell maintaining a constant concentration. No evidence for a direct effect of vitamin D on the serosal pump has been published. From work described in this thesis there is a suggestion that vitamin D_3 is not directly involved at this pump, since when the transport of rachitic duodena was increased over two fold by sodium taurochenodeoxycholate, the pump was able to maintain the intracellular Ca^{2+} at a constant level. Also in the experiment where ethacrynic acid was used to inhibit the serosal pump the vitamin D_3 effect was not eliminated. The serosal pump probably requires an efficient source of metabolic energy. When in vitro intestinal everted sacs take up Ca^{2+} , it is invariably found that the vitamin D_3 -treated sac accumulates more Ca^{2+} within the cell than the rachitic sac. These observations are contrary to the in vivo findings where equal amounts accumulate in the cells. Thus the lack of adequate oxygen and fluid supply to the serosal surface of the in vitro preparation may partially inhibit the serosal pump, causing the accumulation of Ca^{2+} within the mucosal cell, particularly the vitamin D_3 -treated cells.

The plasma to cell and cell to lumen transfers of Ca^{2+} are very small in comparison to the lumen to plasma flux.

To summarise, the postulated mechanism for the effect of vitamin D_3 on Ca^{2+} translocation across the mucosal cell of the duodenum of the chick is (1) a facilitated diffusion of Ca^{2+} across the microvilli enhanced by the CaBP either within or inside the villus membrane, (2) the absorbed Ca^{2+} reaches an equilibrium between cytoplasmic and mitochondrial compartments and this equilibrium is influenced by CaBP. In the absence of CaBP, mitochondria tend to accumulate abnormal amounts of Ca^{2+} , (3) the Ca^{2+} in the cytoplasmic compartment is available to the serosal pump that pumps Ca^{2+} out of the cell against a concentration gradient into the extracellular fluid, and this pump may not be directly influenced by vitamin D (fig. 14).

FIGURE 14

A model for calcium transport through the duodenum mucosal cell of the small intestine of chick

The left hand side of the diagram depicts the synthesis of calcium binding protein and the right hand side shows the mechanism for calcium transport through the cell.

ATP	= adenosine triphosphate
BM	= basement membrane
CaBP	= calcium binding protein
Ca ²⁺	= calcium ion
D	= desmosomes
1, 25 DHCC	= 1,25 dihydroxycholecalciferol
DNA	= deoxyribonucleic acid
ECF	= extracellular fluid
ER	= endoplasmic reticulum
GA	= golgi apparatus
IJ	= intermediary junction
IS	= interstitial space
L	= lysosomes
LM	= lateral membrane
LU	= lumen
M	= mitochondria
MT	= microtubules
MV	= microvilli
N	= nucleus
Na ⁺	= sodium ion
RNA	= ribonucleic acid
TJ	= tight junction
TW	= terminal web

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PUBLICATIONS

- 1 Hamilton, J.W. and Holdsworth, E.S. Calcium transport by the chick small intestine. The role of vitamin D₃, the mitochondria and binding protein. Paper read during Conference on Calcium and Calcified Tissue Metabolism, Melbourne, August, 1969. Abstract published in Calcif. Tissue Res. 4, 284
- 2 Hamilton, J.W. and Holdsworth, E.S. (1970) The release of ⁴⁵Ca from mitochondria of chicken intestinal mucosa by calcium binding protein. Biochem. Biophys. Res. Commun. 40, 1325

THE RELEASE OF ^{45}Ca FROM MITOCHONDRIA OF CHICKEN

INTESTINAL MUCOSA BY CALCIUM BINDING PROTEIN

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Received August 3, 1970

SUMMARY: Mitochondria were isolated from intestinal mucosal cells, loaded in vivo with ^{45}Ca . The specific calcium binding protein (CaBP), whose synthesis is induced by vitamin D, caused the release of ^{45}Ca from these mitochondria. Similar extracts prepared from vitamin D deficient chicks had no effect on ^{45}Ca release. The effects of vitamin D on calcium translocation can be explained by the greater "turnover" of Ca in mitochondria when CaBP is present.

In 1966 Wasserman and Taylor (1) reported the existence of a protein in the intestinal mucosa of vitamin D-replete chicks that was present in much smaller amounts in rachitic chicks and this protein had the property of combining with Ca ions in vitro. This calcium binding protein (CaBP) appeared in the mucosal cells within 5 hr. of giving 5,000 IU of vitamin D₃ to rachitic chicks (2) and the time course of appearance of CaBP was correlated with the increased rate of translocation of Ca which followed treatment with vitamin D. No explanation of the role of CaBP has been put forward.

Hamilton and Holdsworth (3, 4) found that most of the Ca of the intestinal mucosa was present in mitochondria. Furthermore, when the intestinal mucosa was loaded with ^{45}Ca by placing ^{45}Ca in the lumen in vivo, leaving for 20 min., washing out the lumen with saline and allowing absorption to take place for a further 20 min., then ^{45}Ca was lost more rapidly from the intestinal mitochondria of vitamin D-replete than from rachitic chicks (4). Therefore it seems possible that mitochondria may be a site of regulation of calcium transport and this paper reports that CaBP causes the release of Ca from mitochondria in vitro.

METHODS

Crossbred cockerels were maintained on a vitamin D-deficient diet from hatching until four weeks of age (5). Vitamin D repletion was done 16 hr. before the isolation of mitochondria or 72 hrs. before the preparation of CaBP, by giving 400 IU of vitamin D₃ by intramuscular injection.

The binding protein was isolated from the duodena of vitamin D₃-deficient (RBP) and from repleted chicks (D₃BP) by the method described by Wasserman, Corradino and Taylor (6) except that the protein content of the extract was reduced by heating at 60° for 10 min. before the ammonium sulphate precipitation step. After dialysis, the preparation was concentrated by ultrafiltration using a UM 10 membrane of an Amicon Diaflo apparatus (Amicon Corp., Cambridge, Mass.) so that 1 ml contained the equivalent of BP from one duodenum. These extracts were tested for their binding activity for Ca by the Chelex method (1, 7).

Mitochondria, loaded in vivo with ⁴⁵Ca, were prepared as previously described (4), suspended in 0.25M sucrose and the protein content adjusted to between 3-5 mg/ml. The mitochondria, 3-5 mg, were stirred at 0° or 30° in 2 ml medium containing; 0.25M sucrose, 10 mg. bovine serum albumin, 30 mM NaCl and 4 mM Tris HCl, pH 7.4. Binding protein when present was added in amounts equivalent to $\frac{1}{8}$, $\frac{1}{4}$, or $\frac{1}{2}$ of the total BP extractable from one duodenum. At zero time and at 5 min. intervals, 0.5 ml samples were withdrawn and filtered through a 0.65 μ , 25 mm diam. Millipore filter. Reduced pressure was applied to the membrane so that the solution passed through in 10 sec. although suction was maintained for 90 sec. The whole membrane and 0.1 ml of the filtrate were counted separately by liquid scintillation counting in the phosphor described by Bray (8). All the results quoted are mean values of 4 observations.

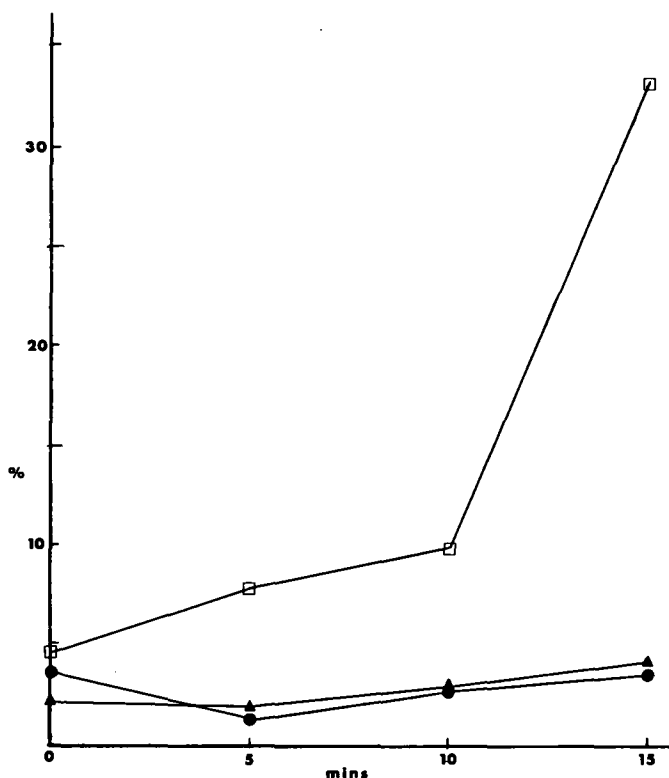


Figure 1. Percent Release of ^{45}Ca from Vitamin D_3 -Replete Mitochondria by CaBP at 30° .

Mitochondria loaded with ^{45}Ca in vivo were prepared from vitamin- D_3 -replete chicks and incubated at 30° in the medium described in the text. Binding protein from rachitic (RBP) and vitamin D_3 -treated chicks (D_3BP) was added in amounts representing half the binding activity of a duodenum. The release of ^{45}Ca is plotted against time of incubation.

●—● Control
 ▲—▲ R.B.P.
 □—□ D_3BP

RESULTS

At 0° , between 3 and 5% of the ^{45}Ca in mitochondria was released to the medium but this release did not increase with time and was not affected by binding - proteins prepared from either vitamin D-deficient or replete chicks. At 30° , CaBP prepared from vitamin D_3 -replete chicks caused the release of ^{45}Ca from mito-

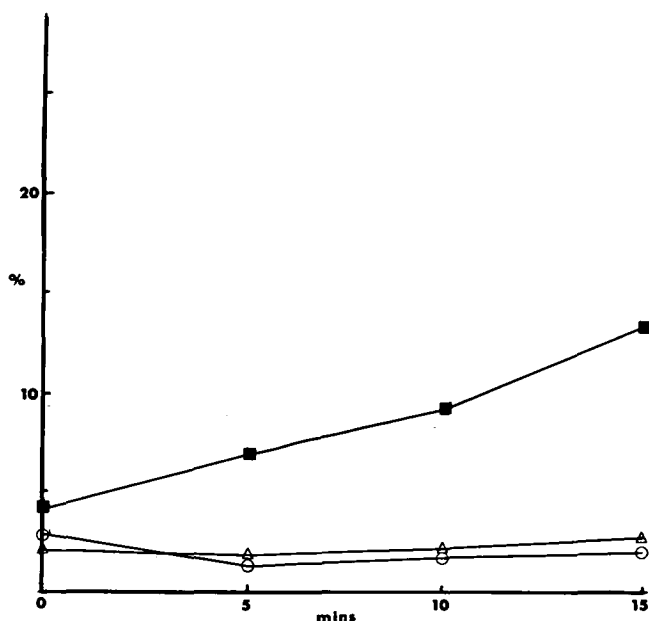


Figure 2. Percent Release of ^{45}Ca from Vitamin D_3 -Deficient Mitochondria by CaBP at 30°

Mitochondria loaded with ^{45}Ca *in vivo* were prepared from vitamin- D_3 -deficient chicks and incubated at 30° in the medium described in the text. Binding protein from rachitic (RBP) and vitamin D_3 -treated chicks (D_3BP) was added in amounts representing half the binding activity of a duodenum. The release of ^{45}Ca is plotted against time of incubation.

—○—○— Control
 —△—△— R.B.P.
 —■—■— D_3BP

chondria isolated from either deficient or replete chicks.

Extracts prepared from duodena of vitamin D_3 -deficient chicks did not cause the release of ^{45}Ca from mitochondria in amounts significantly different from the control mitochondria. The release of ^{45}Ca caused by CaBP increased with increasing time of incubation as shown in Figs. 1 and 2, and was dependent on the amount of CaBP added (Table 1).

DISCUSSION

During the translocation of Ca across the intestine, at any

given time most of this Ca is present in mitochondria (4) and has to be released before it can cross the cell membranes to enter the extracellular fluid. The experiments reported here show that CaBP can cause such a release and that the process depends on the amount of CaBP present, on the length of time it acts and on temperature. Ebel et al. (2) found by fluorescent-antibody techniques, that CaBP appeared to be located at the brush border but in order to affect mitochondria the CaBP is more likely to be present in the cytoplasm. In support of this idea of a cytoplasmic location, it was found that after homogenation of mucosal cells in 0.25M sucrose and differential centrifugation, the CaBP was mostly in the 100,000 X g supernatant (4). The specific CaBP is made only when birds received vitamin D₃ and extracts from deficient birds contain no CaBP nor do these extracts cause release of ⁴⁵Ca from mitochondria. The CaBP released more ⁴⁵Ca from the mitochondria of replete than from the mitochondria of deficient chicks, (compare Figs. 1 and 2). This may have been due to the presence of CaBP or similar transport proteins in these repleted mitochondria. Thus the effect of vitamin D₃ on Ca transport across the intestine seems to be mediated by CaBP, which seems to increase "turnover" of Ca in mitochondria. These results confirm and extend the findings in our previous paper (4) where the overall implications for calcium absorption are discussed.

Table 1. The Effect of conc. of CaBP on ⁴⁵Ca Release from Mitochondria

Mitochondria, loaded in vivo with ⁴⁵Ca, were isolated from vitamin D₃-replete chicks and were incubated at 30° for 15 min. with different amounts of CaBP. The ⁴⁵Ca released was measured as described in the text.

<u>Amount of CaBP (mg)</u>	<u>% of original ⁴⁵Ca released</u>
none	2.9
1.5	5.4
3.0	32.0
6.0	64.0

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CALCIUM TRANSPORT BY THE CHICK SMALL INTESTINE
THE RÔLE OF VITAMIN D₃, THE MITOCHONDRIA AND BINDING PROTEIN

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The in vivo transport of ^{45}Ca by duodenal loops of the chick is increased at least three fold in vitamin D₃ treated compared to deficient chicks. The ^{45}Ca accumulated by the mucosal cells of the two groups during in vivo absorption was the same. Careful fractionation in sucrose gradients located approximately 70% of the ^{45}Ca in mitochondria with 10-20% in nuclei, 1-2% in microsomes and only traces of ^{45}Ca in the soluble fraction. This distribution could be an artefact since it was found the cell homogenates in 0.25M Sucrose at 0° would incorporate ^{45}Ca in amounts normally present in mucosal cells, into mitochondria and nuclei. Inhibitors were sought that would prevent uptake of ^{45}Ca at 0° but would not cause its release from mitochondria. Antimycin A and Warfarin were found to be suitable and when the cells were homogenised in the presence of these two inhibitors (combined) the distribution was similar to that above. Thus it was concluded the ^{45}Ca being transported occurred in mitochondria and very little was in the cytoplasm.

The calcium-binding or carrier protein discovered by Wasserman and Taylor was found to be located mainly in the soluble part of the cell with small amounts in the organelles. This binding protein was able to release ^{45}Ca from mitochondria "loaded" in vivo of both deficient or vitamin D₃-replete chick intestine. A scheme showing how the "binding-protein" could increase ^{45}Ca transport will be presented.

REFERENCE:

R. H. Wasserman and A. N. Taylor. Science, 152, (1966) 791 and
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